

# **In Vitro Antimicrobial Activity of Stem Bark Extract of *Azadirachta indica* A. (JUSS) Against Antibiotic Resistant *Salmonella enterica* Serovar Typhi**

**Bobai Mathew<sup>1,\*</sup>, Yabaya Amos<sup>1</sup>, Orukotan Ayodeji Abimbola<sup>1</sup>, Joshua Istifanus Anekoson<sup>2</sup>, Amos Alvan<sup>3</sup>**

<sup>1</sup>Department of Microbiology, Faculty of Science, Kaduna State University, Kaduna, Nigeria

<sup>2</sup>Department of Community Medicine, Faculty of Medicine, Kaduna State University, Kaduna, Nigeria

<sup>3</sup>Department of Biochemistry, Faculty of Science, Kaduna State University, Kaduna, Nigeria

## **Email address:**

[bobaimathkaya@yahoo.com](mailto:bobaimathkaya@yahoo.com) (B. Mathew), [amosyabaya@gmail.com](mailto:amosyabaya@gmail.com) (Y. Amos), [bimborukot@yahoo.com](mailto:bimborukot@yahoo.com) (O. A. Abimbola), [distifanus@yahoo.com](mailto:distifanus@yahoo.com) (J. I. Anekoson), [alvanmos188@yahoo.com](mailto:alvanmos188@yahoo.com) (A. Alvan)

\*Corresponding author

## **To cite this article:**

Bobai Mathew, Yabaya Amos, Orukotan Ayodeji Abimbola, Joshua Istifanus Anekoson, Amos Alvan. In Vitro Antimicrobial Activity of Stem Bark Extract of *Azadirachta indica* A. (JUSS) Against Antibiotic Resistant *Salmonella enterica* Serovar Typhi. *American Journal of Laboratory Medicine*. Vol. 2, No. 6, 2017, pp. 163-171. doi: 10.11648/j.ajlm.20170206.18

**Received:** March 28, 2017; **Accepted:** April 17, 2017; **Published:** November 28, 2017

**Abstract:** Typhoid fever treatment failure due to emergence of antibiotics resistant *Salmonella enterica* serovar Typhi has become a matter of concern to public health sector. This study was aimed at investigating the *in vitro* antimicrobial activity of stem bark extracts of *Azadirachta indica* A. (JUSS) against antibiotic resistant *Salmonella enterica* serovar Typhi isolates from stool samples of patients manifesting clinical features of typhoid fever. Fortyseven (47) isolates were identified as *Salmonella* sp. using conventional biochemical test, Microbact<sup>TM</sup> 12A/12E Gram-negative kits, and confirmed molecularly using ompC primers. Twelve (12) isolates out of the 47 were *Salmonella enterica* serovar Typhi identified serologically and confirmed molecularly using STY4220 primers. Antibiotics resistance profile of the *Salmonella enterica* serovar Typhi isolates showed that 12 (100%) were resistance to amoxicillin and amoxicillin/clavulanic acid, 9 (75%) resistance to tetracycline, cotrimoxazole and cefotaxime, 6 (50%) resistance to ceftriaxone, 3 (25%) resistance to nitrofurantoin, 2 (17%) resistance to chloramphenicol, 1 (8%) resistance to ofloxacin and ciprofloxacin; while 12 (100%) were sensitive to nalidixic acid and imipenem. Qualitative phytochemical and spectroscopic analysis of *Azadirachta indica* stem bark extracts revealed the presence of tannins, alkaloids, flavonoids, glycosides, saponins, phenolic and other organic compounds. At concentrations of 25-400 mg/ml, potency test of acetone and ethanolic bark extracts of the plant against the *Salmonella enterica* serovar Typhi isolates produced diameter zones of growth inhibition ranges from 18-35 mm and 15-31 mm respectively. The minimum inhibitory concentrations values of the extracts against the isolates also ranged from 50-100 mg/ml. It may be concluded from this results that the bark extracts of *Azadirachta indica* A. (JUSS) have good growth inhibitory effects against the antibiotics resistant *Salmonella enterica* serovar Typhi and can be developed further for chemotherapeutic application.

**Keywords:** *In vitro*, Antibiotic Resistant, *Azadirachta indica*, *Salmonella enterica* Serovar Typhi

## **1. Introduction**

Antibiotic resistance, dangerous side effects of synthetic drugs and their high costs of production have become a serious threat to public health. One of the diseases that poses serious threat to the health of millions people worldwide is

typhoid fever. This fever mostly responds slowly to ampicillin, amoxicillin, cotrimoxazole or trimethoprim alone. However, ciprofloxacin, ofloxacin and perfloxacin are the most widely used antimicrobial agents, but ciprofloxacin resistant salmonellae have emerged and have been a matter of concern [1, 2]. Despite efforts to control the emergence and spread of drug resistance, the situation continues to worsen

[3]. Also, due to the alarming spread of chloramphenicol resistant *Salmonella enterica* serovar Typhi throughout the world, newer antibiotics with good *in vivo* activity against *Salmonella enterica* serovar Typhi are needed [4].

Natural products, such as plants extracts, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discovery because of the unmatched availability of chemical diversity. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy, where thousands of phytochemicals from plants are found literally as safe and broadly effective alternatives with less adverse effect [5].

According to World Health Organization (WHO), medicinal plants would be the best source of a variety of drugs. Nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries [5, 6]. It is revealed that herbal drugs are relatively safe and exhibit a remarkable efficacy in treatment and are clinically safer alternatives to the synthetic antibiotics [7]. Hence, researchers are increasingly turning their attention to herbal products, looking for new leads to develop effective and better drugs against multidrug resistant pathogenic microorganisms [8].

Earlier studies on *Azadirachta indica* have showed that it contains active substances with multiple medicinal properties such as antibacterial, antifungal, antimalarial, antiviral, antidiabetic, antipyretic, and antifertility activity [9, 10]. The chemical constituents of the plant consist of many bioactive compounds including alkaloids, flavanoids, triterpenoids, phenolic compounds, carotenoids, sterioids and ketones [11]. The presence of phytochemicals such as alkaloids, tannins, triterpenoids, sterioids, glycosides, flavonoids and phenolic compounds in plant extracts supports their traditional use as therapeutic agents [8, 12]. Plant based traditional medicine continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicine for their primary health care [6, 8]. Hence, there is the need for natural and reliable, affordable, effective, and comparatively safe drugs against multidrug resistant pathogens to be developed.

Therefore, this study aimed at investigating the *in vitro* antimicrobial activity of stem bark extract of *Azadirachta indica* A. (JUSS) against antibiotic resistant *Salmonella enterica* serovar Typhi.

## 2. Materials and Methods

### 2.1. Collection of Samples

A total of 220 stool samples were collected from patients manifesting clinical features of typhoid fever. The samples were collected from five hospitals in Kaduna metropolis. An ethical clearance was obtained from Kaduna state ministry of health. Clean sterile wide-necked leak proof screw- cap bottles were used to collect the samples from the patients with the help of the hospital Laboratory Staff. The samples collected were then transported in thermo flasks packed with ice to Kaduna State

University Microbiology Laboratory for analysis.

### 2.2. Isolation of *Salmonella enterica* Serovar Typhi from Clinical Specimens

All stool samples collected were cultured aerobically for isolation of *Salmonella* sp. in the laboratory as described by Vallis *et al* [13] and Cheesbrough [14]. To isolate the organism, 1ml of each stool sample collected was directly inoculated into 9ml of Selenite Cysteine broth at the point of collection in the hospital laboratory. The samples were transported in an ice pack thermo-flask to the Department of Microbiology Laboratory, Kaduna State University, Kaduna and incubated aerobically for 12 hours at 37°C. After the 12 hours enrichment period, the samples were heavily inoculated on *Salmonella Shigella* agar plates and incubated for 24 hours at 37°C. Black dotted center colonies with or without transparent borders were identified and subcultured on MacConkey agar plates. Non-lactose fermenters colonies were subculture into nutrient agar slant and kept at 4°C for further analysis.

### 2.3. Biochemical, Serological and Molecular Identification of *Salmonella enterica* Serovar Typhi

The Isolates were subjected to conventional biochemical identification test following the procedure as described by Ochai and Kolhatkar [15] and Cheesbrough [14]. These includes Gram staining, motility, indole, urease, citrate, triple sugar iron and oxidase test. Microbact™ 12A/12E (Oxoid) Gram - negative identification kit was further used for the identification of the presumptive *Salmonella* species isolates following the manufacturer's protocol.

The isolates were subjected to molecular identification using the ompC and STY4220 primers. DNA extraction was carried out using EasyPure Bacteria DNA kit, following the protocol for gram negative bacteria supplied by the manufacturer. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer. DNA samples were stored at -20°C for PCR analysis.

Polymerase chain reaction (PCR) amplification was carried out using the primer sets for ompC gene (Forward - 5' ATC GCT GAC TTA TGC AAT CG 3', Reverse - 5' CGG GTT GCG TTA TAG GTC TG 3') to detect *Salmonella* species. *Salmonella Typhimurium* 14028, a reference strain obtained from Nigerian Institute of Medical research Yaba, was used as positive control.

Serological test for the detection of *Salmonella enterica* serovar Typhi was carried out using agglutinating antisera according to Srirangaraj *et al* [16].

For molecular identification of *Salmonella enterica* serovar Typhi, the same procedure as described above was followed, but primer sets for STY 4220 gene (Forward - 1 5' AGT ATC ACC GCC TGC CAT CT 3', Reverse - 1 5' CGG CAG CAA TTG GCT CAT AC 3') were used.

### 2.4. Determination of In Vitro Antimicrobial Activity of *Salmonella enterica* Serovar Typhi Strains

*In vitro* Sensitivity of the pure culture of molecularly identified *S. enterica* serovar Typhi strains to different antibiotics was determined using Kirby-Bauer disc diffusion technique as described by Arora [2]. *Escherichia coli* ATCC 25922 from Nigerian Institute of Medical Research (NIMR), was used as control. Interpretation of the isolates as sensitive or resistant was based on Clinical and Laboratory Standards Institute guidelines [17].

### 2.5. Preparation of *Azadirachta indica* A. (JUSS) Ethanolic and Acetone Extracts

The *Azadirachta indica* plant from which the stem bark was obtained was authenticated by a Taxonomist at the herbarium unit of the department of Biological Science, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria and the plant specimen with voucher number 900151 was deposited for future reference. The *Azadirachta indica* stem bark was shade dried in a clean laboratory cabinet. The dried plant material was pounded in a mortar, followed by dry-milling with an electric blender and then sieved into fine powder using 20 µm mesh size. Twenty-five gram (25 g) of the processed fine powder sample was soaked in separate 250 ml of ethanol and acetone in sterile 500 ml conical flasks and kept for about 72 hours with periodic shaken. The contents were filtered first using clean muslin cloths, followed by Whatman's No.1 filter paper. The filtrates were concentrated under reduced pressure at 37°C using rotatory evaporator and the extracts were powdered and kept refrigerated for further work.

### 2.6. Physicochemical Analysis of the Plant Extracts

The characteristics of extracts like pH, color and consistency of the extracts were observed. The extracts were subjected to qualitative phytochemical analysis to determine the presence of saponins, tannins, phenolic compounds, anthraquinones, cardiac glycosides, alkaloids, and flavonoids, using standard procedures described by Sofowara [18] Trease and Evans [19]; and Radhika *et al* [8]. Spectroscopic analysis of the extracts was carried out using Fourier Transform Infra-Red (FTIR) spectrophotometer for the presence of organic compounds and interprets the results according to Morrison and Boyd [20].

### 2.7. In Vitro Antibacterial Potency of the Extracts Against *Salmonella enterica* Serovar Typhi Strains

The antimicrobial potency of the plants extracts against all the twelve (12) antibiotics resistant *Salmonella enterica* serovar Typhi strains was determined using agar-well diffusion method described by Ochai and Kolhatkar [15]; and Cheesbrough [14]. Acetone and ethanolic extracts of dried *Azadirachta indica* stem bark were reconstituted in 2% Dimethyl Sulfoxide (DMSO) to make concentrations of 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml. 20 ml of sterile molten Muller-Hinton agar was poured into each

of the petri plates and allowed to solidified. Overnight broth cultures of each pure isolate was then adjusted by comparing with 0.5 Mcfarland turbidity standard (Density of  $1.0 \times 10^8$  cells/ml) against a light background. Sterile cotton wool swab was dipped into the suspension, removed and streaked uniformly on the surface of Muller-Hinton culture plates. After 5 minutes, 6mm cork borer was used to make wells on the inoculated culture plates and 0.5 ml each of the reconstituted extract concentrations were loaded into the wells using sterile micropipettes. The plates were kept for 2 hours for extracts diffusion and then incubated for 24 hours at 37°C. 1mg/ml of ciprofloxacin and chloramphenicol were used as positive controls; while 2% DMSO, ethanol, and acetone were used as negative controls. Zones of growth inhibition were measured with a meter rule and the results recorded in millimeter (mm).

### 2.8. Determination of Minimum Inhibitory Concentration (MIC) of the Plant Extracts

The concentrations of acetone and ethanolic stem bark extracts that showed antimicrobial activity were used for the determination of the minimum inhibitory concentrations (MIC). Acetone and ethanolic stem bark extracts were reconstituted in 2% DMSO to get concentration of 500 mg/ml as stock solution. 400 mg/ml, 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml, were prepared from the stock solution using Muller-Hinton broth as the diluent. 1 ml each of these prepared concentrations was transferred into test tubes containing 1ml of Muller-Hinton broth followed by 1ml of the standardized isolates suspension of  $10^8$  cfu/ml density. The tubes were incubated aerobically at 37 °C for 24 hours. The lowest concentrations of the extracts which showed no visible growth were recorded as the MIC concentrations of the extracts.

## 3. Results

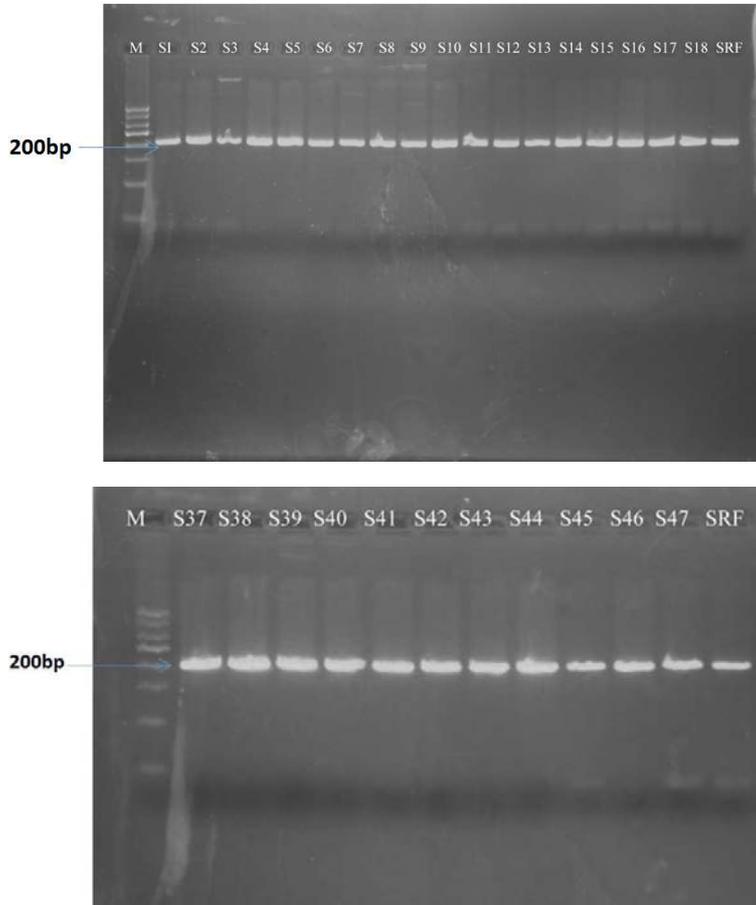
### 3.1. Biochemical, Serological and Molecular Identification of *Salmonella enterica* Serovar Typhi

Table 1 shows that Fortyseven (47) isolates were identified as *Salmonella* sp. after the conventional biochemical identification, Microbact™ 12A/12E Gram - negative identification test and PCR amplification which was carried out using the set of primer ompC gene (Forward - 5' ATC GCT GAC TTA TGC AAT CG 3' and Reverse - 5' CGG GTT GCG TTA TAG GTC TG 3'). The PCR products confirmed the presence of 204bp ompC gene of *Salmonella* sp. (Figure 1).

Out of 47 isolates, 12 were identified serologically as *Salmonella enterica* serovar Typhi and confirmed through PCR using a set of primer for STY4220 gene (Forward - 1 5' AGT ATC ACC GCC TGC CAT CT 3' and Reverse - 1 5' CGG CAG CAA TTG GCT CAT AC 3') (Figure 2).

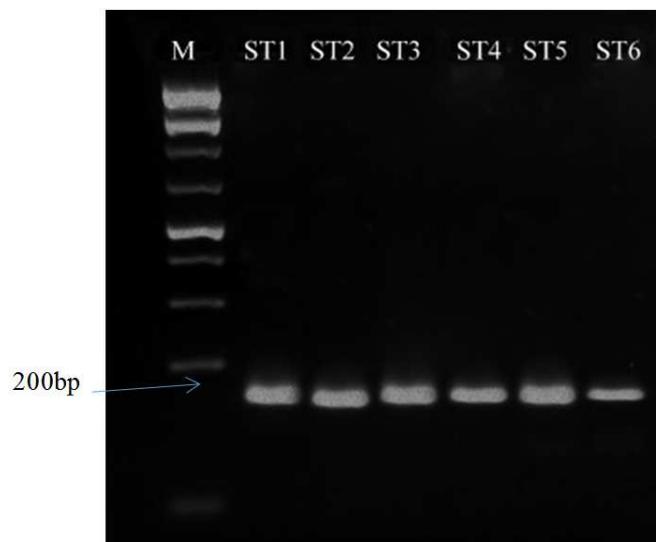
**Table 1.** Distribution of *Salmonella* sp. and *Salmonella enterica* serovar Typhi Among Patients of Different Age Groups.

| S/N | Salmonella Isolate                       | ≤10 yrs | 11-20 yrs | 21-30 yrs | 31-40 yrs | 41-above yrs | Total Isolate |
|-----|--|---------|-----------|-----------|-----------|--------------|---------------|
| 1   | <i>Salmonella</i> sp.                    | 20      | 05        | 04        | 05        | 13           | 47            |
| 2   | <i>Salmonella enterica</i> serovar Typhi | 04      | 02        | 01        | 00        | 05           | 12            |



**Figure 1.** Gel Electrophoresis Showing 204 bp *ompC* genes of *Salmonella* sp. on a 50kb Marker.

M = Lane for DNA molecular weight marker. The first bottom electrophoresis band of the marker on lane M = 50bp; and the molecular weight increases by 50bp on each additional band upward the lane.  
 Lane S1 to S47 showed positive (bands) to *ompC* gene primers – this indicated that S1 to S47 are *Salmonella* sp. isolates.  
 SRF = *Salmonella* sp. reference isolate (*Salmonella Typhimurium* 14028).



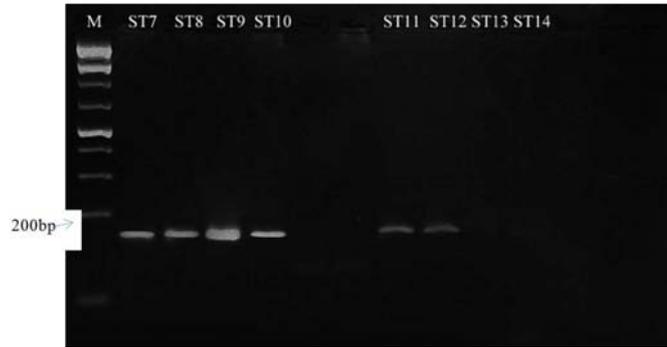


Figure 2. Gel electrophoresis showing bands of 176bp STY 4220 genes of *Salmonella enterica* serovar Typhi on a 1000bp marker.

M = 1kb DNA molecular weight marker Lane. The first bottom electrophoresis band of the marker on lane M = 100bp; and the molecular weight increases by 100bp for each additional band upward the lane. Lane ST1 to ST12 showed positive (bands) to STY4220 gene primers - this indicated that ST1 to ST12 are *Salmonella enterica* serovar Typhi isolates. ST13 and ST14 showed negative (no bands) to STY4220 gene primers - this indicated that ST13 and ST14 are not *Salmonella enterica* serovar Typhi Isolates).

3.2. In Vitro Activity of Antibiotics Against *Salmonella enterica* Serovar Typhi Strains

The drugs resistance profile (Table 2 and 3) revealed multidrug resistance pattern where the isolates exhibited high resistance to Amoxicillin and amoxicillin/clavulanic acid 12 (100%), followed by tetracycline, cotrimoxazole and cefotaxime 9 (75%), ceftriaxone 6 (50%), nitrofurantoin 3 (25%), chloramphenicol 2 (17%), ofloxacin and ciprofloxacin 1 (8%) and, nalidixic acid and imipenem 0 (0%).

Table 2. In vitro Activity of Conventional Antimicrobial Agents Against *Salmonella enterica* serovar Typhi Isolates Strains.

| S/N | Antibiotic | Disc content(µg) | ST1 | ST2 | ST3 | ST4 | ST5 | ST6 | ST7 | ST8 | ST9 | ST10 | ST11 | ST12 | %R  |
|-----|------------|------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|------|------|-----|
| 1   | AMX        | 25               | R   | R   | R   | R   | R   | R   | R   | R   | R   | R    | R    | R    | 100 |
| 2   | AMC        | 3                | R   | R   | R   | R   | R   | R   | R   | R   | R   | R    | R    | R    | 100 |
| 3   | CRO        | 30               | I   | I   | R   | I   | R   | R   | R   | S   | I   | S    | R    | R    | 50  |
| 4   | CEF        | 30               | R   | R   | R   | R   | S   | R   | R   | R   | R   | S    | S    | R    | 75  |
| 5   | C          | 30               | S   | S   | S   | S   | I   | S   | I   | R   | S   | I    | R    | S    | 17  |
| 6   | TET        | 25               | R   | R   | S   | R   | R   | S   | R   | R   | R   | R    | R    | S    | 75  |
| 7   | COT        | 25               | R   | R   | R   | S   | S   | R   | R   | R   | R   | S    | R    | R    | 75  |
| 8   | NA         | 30               | S   | S   | S   | S   | S   | S   | S   | S   | S   | S    | S    | S    | 00  |
| 9   | OFL        | 5                | S   | S   | S   | S   | S   | S   | S   | S   | S   | S    | R    | S    | 08  |
| 10  | CIP        | 5                | I   | I   | S   | I   | S   | I   | S   | R   | I   | S    | I    | S    | 08  |
| 11  | IPM        | 10               | S   | I   | S   | S   | S   | S   | I   | I   | S   | I    | I    | S    | 00  |
| 12  | NIT        | 300              | S   | S   | S   | S   | R   | S   | R   | R   | S   | S    | I    | S    | 25  |
|     | % R        |                  | 42  | 42  | 42  | 33  | 42  | 42  | 58  | 67  | 42  | 25   | 58   | 42   |     |

AMX=Amoxicillin (25 µg), AMC=Amoxicillin/clavulanic acid (30 µg), TET= Tetracycline (25 µg), COT= Cotrimoxazole (25 µg), C= Chloramphenicol (30 µg), CEF=Cefotaxime (30 µg), CRO= Ceftriaxone (30 µg), NIT= Nitrofurantoin (300 µg), NA= Nalidixic acid (30 µg), CIP= Ciprofloxacin (5 µg), OFL= Ofloxacin (5 µg),% R = percentage resistance, and ST1-ST12=*Salmonella enterica* serovar Typhi isolates

Table 3. Resistance Pattern of *Salmonella enterica* serovar Typhi Isolates Against the Conventional Antimicrobial Agents.

| Isolate code | Resistance Pattern                   |
|--------------|--------------------------------------|
| ST1          | AMX, AMC, CEF, TET, COT              |
| ST2          | AMX, AMC, CEF, TET, COT              |
| ST3          | AMX, AMC, CRO, CEF, COT              |
| ST4          | AMX, AMC, CEF, TET                   |
| ST5          | AMX, AMC, CRO, TET, NIT              |
| ST6          | AMX, AMC, CRO, CEF, COT              |
| ST7          | AMX, AMC, CRO, CEF, TET, COT, NIT    |
| ST8          | AMX, AMC, CEF, C, TET, COT, CIP, NIT |
| ST9          | AMX, AMC, CEF, TET, COT              |
| ST10         | AMX, AMC, TET                        |
| ST11         | AMX, AMC, CRO, C, TET, COT, OFL      |
| ST12         | AMX, AMC, CRO, CEF, COT              |

AMX=Amoxicillin (25 µg), AMC=Amoxicillin/clavulanic acid (30 µg), TET= Tetracycline (25 µg), COT= Cotrimoxazole (25 µg), C= Chloramphenicol (30 µg), CEF=Cefotaxime (30 µg), CRO= Ceftriaxone (30µg), NIT= Nitrofurantoin (300 µg), NA= Nalidixic acid (30 µg), CIP= Ciprofloxacin (5 µg), OFL= Ofloxacin (5 µg), and ST1-ST12=*Salmonella enterica* serovar Typhi isolates.

### 3.3. Physicochemical Characteristics of Acetone and Ethanolic Stem Bark Extracts of *Azadirachta Indica A. (JUSS)*

Qualitative phytochemical and spectroscopic analysis of acetone and ethanolic stem bark extracts of *Azadirachta indica A.(JUSS)* revealed the presence of tannins, alkaloids, flavonoids, glycosides, phenolic compounds, saponins, ketones, carboxylic acids, aromatic compounds, amides, proteins, aldehydes and aliphatic compounds; while the pH of the acetone and ethanolic extracts were 4.85 and 4.75 respectively; with both extracts brown in color and powdered (solid) form the physicochemical analysis (Table 4).

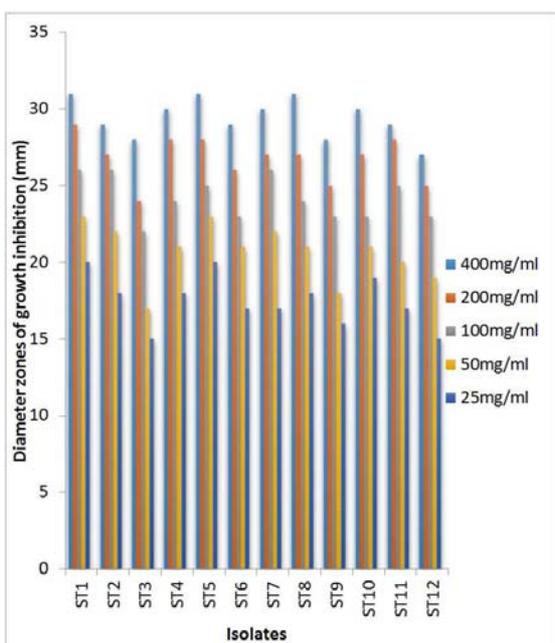
**Table 4.** Physicochemical Characteristics of *Azadirachta indica* Stem Bark Extracts.

| S/N | Extract | Phytochemicals Present |       |       |     |     |      |      |      |     | Functional Group Analysis Using Spectroscopy |   |   |  |
|-----|---------|------------------------|-------|-------|-----|-----|------|------|------|-----|--|---|---|--|
|     |         | pH                     | Col.  | Cons. | TA. | SA. | GLY. | ALK. | PHE. | AN. | FL.  | FNG.  | COMP.   | MOV.   |
| 1   | Ethanol | 4.75                   | Brown | solid | +   | +   | +    | +    | +    | -   | +  | O-H<br>O-H<br>C=O<br>NO <sub>2</sub><br>CH <sub>3</sub> , CH <sub>2</sub> & CH<br>C-O<br>N-H<br>C-H | Phenol<br>Phenol<br>Carboxylic, Saturated ketones & aromatic<br>Aromatic, Aliphatic & monosubstitution<br>Alkanes<br>Phenol<br>Proteins<br>Aldehyde | Diametric<br>Stretching<br>Stretching<br>Stretching<br>Stretching<br>Stretching<br>Polymeric<br>Stretching |
| 2.  | Acetone | 4.85                   | Brown | solid | +   | +   | +    | +    | +    | -   | +  | SFE   | SFE   | SFE  |

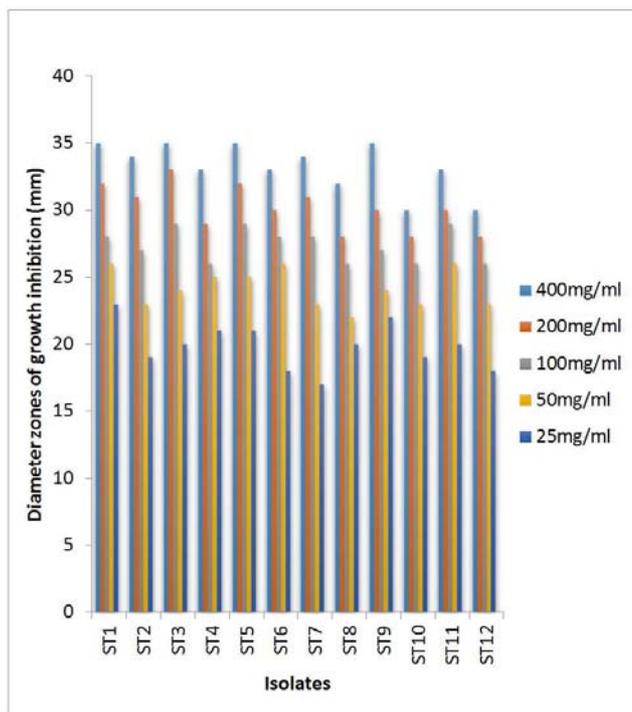
Col. = Colour, Cons. = Consistency, TA. = Tannins, SA = Saponins, ALK. = Alkanoids, PHE. = Phenolic compounds, GLY. = Glycosides, AN. = Anthraquinones, FL. = Flavonoids, FNG. = Functional groups, COMP = Compound, MOV. = Mode of vibrations, + = Present, - = Absent, SFE = Same findings as in ethanol extract.

### 3.4. In vitro Antimicrobial Activity of Bark Extracts of *Azadirachta indica A. (JUSS)* Against Antibiotic Resistant *Salmonella enterica* Serovar Typhi Strains

Acetone and ethanolic extracts showed measurable diameter zones of inhibition at 25-400 mg/ml. The acetone extract had 18-35 mm, while ethanol extract had 15-31 mm diameter zones of inhibition. The antibacterial activity of the extracts against the 12 multi drug resistant isolates is shown in Figure 3 and 4.



**Figure 3.** In vitro Antimicrobial Activity of Ethanol Stem Bark Extract of *Azadirachta indica A. (JUSS)* Against Antibiotic Resistant *Salmonella enterica* serovar Typhi Strains.



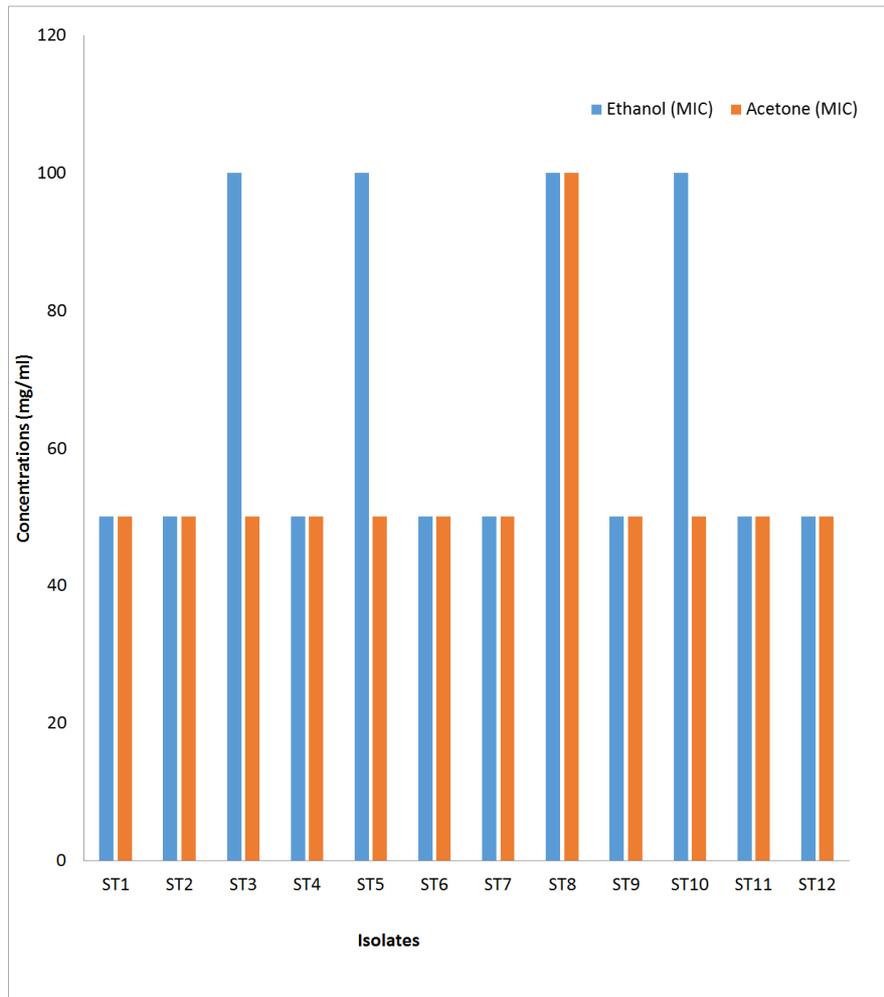
**Figure 4.** In vitro Antimicrobial Activity of Acetone Stem Extract of *Azadirachta indica A. (JUSS)* Against Antibiotic Resistant *Salmonella enterica* serovar Typhi Strains.

### 3.5. Minimum Inhibitory Concentration of the Stem Bark Extracts of *Azadirachta indica A. (JUSS)* Against Antibiotic Resistant *S. enterica* Serovar Typhi Strains

The minimum inhibitory concentrations (MIC) value of the ethanol extract was found to be 50 mg/ml against ST1, ST2, ST4, ST6, ST7, ST9, ST11, and ST12 whereas MIC

value against ST3, ST5, ST8, and ST10 was found to be 100 mg/ml. The minimum inhibitory concentrations (MIC) value of the acetone extract was found to be 50 mg/ml against ST1,

ST2, ST3, ST4, ST5, ST6, ST7, ST9, ST10, ST11, and ST12, whereas MIC value against ST8 was 100 mg/ml (Figure 5).



**Figure 5.** In vitro Minimum Inhibitory Concentration (MIC) of Ethanol and Acetone Stem Bark Extract of *Azadirachta indica* A. (JUSS) Against Antibiotics Resistant *Salmonella enterica* serovar Typhi Strains.

## 4. Discussion

In this study, 47 isolates of *Salmonella* sp. were identified using conventional biochemical tests, Microbact™ 12A/12E Gram-negative identification system, and confirmed using molecular identification through chromosomal DNA extraction using Easypure bacteria DNA kit and PCR, in which the *Salmonella* ompC gene codes for a major outer membrane protein (omp) in the *Salmonella* genus. Ngan *et al* [21] used the ompC gene, which is described by Alvarez *et al* [22], as a *Salmonella*-genus-specific protein for *Salmonellae* identification. Out of the fortyseven *Salmonella* sp., 12 *Salmonella enterica* serovar Typhi were identified molecularly using STY4220 Primers. *Salmonella enterica* serovar Typhi, *Salmonella paratyphi* A, B and C are primarily human pathogens. The infective dose of most serotypes including *Salmonella enterica* serovar Typhi varies from  $10^6$  to  $10^9$  cells [2]. This study showed a spread of *Salmonella enterica* serovar Typhi which is responsible for

typhoid or enteric fever across the study population, indicating endemicity of these infections in Kaduna metropolis. In Nigeria, enteric fever cause by *Salmonella enterica* serovar Typhi is not only endemic [23] but constitute a great socio-medical problem [24]; being responsible for many cases of pyrexia of unknown origin, high morbidity and mortality [25, 26]. Isolation of *Salmonella enterica* serovar Typhi from the patients' stool samples indicate the presence of salmonellae in the intestinal tract of the hosts as either carriers or infectious persons. Arora [2] recorded that *Salmonella* sp. are shed in the faeces throughout the course of the disease and even in convalescence, with varying frequency, and that during the first week of the illness, salmonellae can be isolated from the faeces in about half the cases and they are most easily isolated between third to fifth week of illness.

Findings in this study revealed a higher incidence multidrug resistant (MDR) *Salmonella enterica* serovar Typhi infections among patients of age group 41 years and above.

Similar findings by Abu *et al.*, Brandis *et al.*, and Kam [27, 28, 29], showed high incidence of typhoid fever causative agent in adult of ages above 40 years old. This however, may be due to the fact that their immune system has been depressed as a result of old age or other diseases.

In this study, all the 12 *Salmonella enterica* serovar Typhi isolates showed multidrug resistance (MDR) to Ciprofloxacin (5 µg), Ofloxacin (5 µg), Tetracycline (25 µg), Cotrimoxazole (25 µg), Amoxicillin (25 µg), Amoxicillin/clavulanic acid (30 µg), Nalidixic acid (30 µg), Ceftriazone (30 µg), Cefotaxime (30 µg), Nitrofurantoin (300 µg), and Chloramphenicol (30 µg) with a record of highest (100%) resistance to Amoxicillin and amoxicillin/clavulanic acid 12 (100%) and zero (0%) resistance to nalidixic acid and imipenem 0 (0%). According to Arora [2] and Srirangaraj *et al.* [16], the resistance of *Salmonella enterica* serovar Typhi isolates to these antimicrobial agents is of clinical significance. Ceftriazone, cefotaxime, and cefepime are of the third generation cephalosporins are effective therapeutic alternatives in multidrug resistant *Salmonella enterica* serovar Typhi infected cases and there is a changing trend in the susceptibility pattern of *Salmonella enterica* serovar Typhi worldwide with the emergence of resistance to fluoroquinolone, and consequently reduced the therapeutic options available. Resistant strains of *Salmonella enterica* serovar Typhi to chloramphenicol and cotrimoxazole have emerged and continue to be of clinical significance [30]. Also, an antibiogram of *Salmonella enterica* serovar Typhi in Pondigari shows resistance of *Salmonella enterica* serovar Typhi to chloramphenicol, cotrimoxazole and nalidixic acid [31]. Transferable drug resistance involves all antibiotics in common use and transfer of drug resistance occurs *in vitro* as well as *in vivo*, but in normal intestines it is inhibited by several factors like anaerobic condition, bile salts, alkaline pH, and abundant anaerobes. In the intestines of persons on oral antibiotics therapy however, transfer occurs readily due to selection pressure provided by the drug. However, because of the overuse of antibiotics in the hospitals, it is said 'hospital is the haven for drug resistant bacteria [2] and most resistance of concern are associated with R-plasmid [2].

Qualitative phytochemical and spectroscopic analyses of the extracts revealed the presence of bioactive compounds. The most important of these bioactive compounds are alkaloids, tannins, saponins, flavonoids and phenolic compounds [8]. Their presence in crude extracts is known to confer antibacterial activity against disease causing microorganisms [12], thus indicating the medicinal value of *Azadirachta indica*. The *in vitro* activity of the acetone and ethanol bark extracts of the *Azadirachta indica* A. (JUSS) against the antimicrobial drug resistant *Salmonella enterica* serovar Typhi strains revealed large diameter zones of growth inhibitions against the tested isolates. These zones of growth inhibitions generally vary depending on extracts concentrations and strains of the tested isolates may depend on the presence of the bioactive ingredients, the strength of these bioactive ingredients and their capacity to diffuse into the agar medium. The minimum inhibitory concentrations

(MIC) of the acetone and ethanol extracts were found to be 100 mg/ml and 50 mg/ml. The values vary depending on the *Salmonella enterica* serovar Typhi strains. Several findings on the chemotherapeutic potentials of plants have shown that plant are sources of antimicrobial compound of value [32]. Beg and Ahmad [33] tested alcoholic extract of *Plumbago zeylanica* against multi-drug resistant clinical isolates of bacteria namely *S. paratyphi*, *S. aureus*, *E. coli*, *Shigella dysenteriae*, and the extract exhibited strong antibacterial activity against all the bacteria tested irrespective of their antibiotic resistance behavior.

It can be deduced from the result obtained in this study that the bark extracts of *Azadirachta indica* A. (JUSS) have good inhibitory effects against antibiotic resistant *Salmonella enterica* serovar Typhi, and can therefore be developed further for chemotherapeutic application.

## 5. Conclusion

In conclusion, *Azadirachta indica* A (JUSS) stem bark extracts showed remarkable *in vitro* antibacterial activity against the antibiotic resistant *Salmonella enterica* serovar Typhi indicating that the extracts of this plant can be used to produce drugs which can effectively combat multidrug resistant strains of *Salmonella enterica* serovar Typhi. This however, requires much more exhaustive studies involving isolation and identification of the specific active phytochemicals against the multidrug resistant *Salmonella enterica* serovar Typhi (MDRST).

## References

- [1] Basudha K., Sanjib K. S., Shyamal K. B., Narayan R. B., Monorama D. and Reba K. Antimicrobial susceptibility patterns of *Salmonella enterica* serotype Typhi in Eastern Nepal. *Journal of Health, Population and Nutrition*, 2007; 25(1): 82-87
- [2] Arora, D. R. and Arora, B. A Text Book of Microbiology. 3<sup>rd</sup> edition, CBS Publishers Pvt. Ltd New Delhi, India. pp: 352, 368-373, 376, 382, 385, 410-412.
- [3] Prescott, L. M, Harley, J. P, and Klein, A. D. Microbiology. 7<sup>th</sup> edition McGraw-Hill, New York, 2008; pp:852-853, 53-54
- [4] Black, J. G. Sterilization and disinfection, microbiology principles and explorations 6<sup>th</sup> Edition. John Wiley and Son, U.S.A. 2005; pp: 347, 355-362.
- [5] Sasidharan, S., Chen, Y., Saravanan, D., Sundran, M. K., and Latha, Y. L. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal on Traditional Complement Alternative Medicine*, 2011; 8(1): 1-10.
- [6] Jyothi, A. and Thomas, D. T. Antibacterial activity of medicinal plant *Cyclea peltata* (Lam) Hooks and Thomas. *Asian Pacific Journal of Tropical Disease*, 2012; S280-S284.
- [7] Ashajyothi, C., Ramachandra, Y. L. and Padmalatha, R. A Review on antibacterial activity of some medicinal plants of Western Ghat. *World Journal of Pharmaceutical Research*, 2012; 1(4): 964-974.

- [8] Radhika, B., Murthy, N and Nirmala, D. Preliminary phytochemical analysis and antibacterial activity against clinical pathogens of medically important Orchid *Cymbidium aloifolium* (L.) SW. *International Journal of Pharmaceutical Sciences and Research*, 2013. 4(10):3925-3931.
- [9] Itelima, J. U. J. and Nwokedi, V. C. Antimicrobial activities of leaf of *Azadirachta indica* (Neem Plant). *West African Journal of Biological Sciences*. 2003; 14:37-42.
- [10] Maragathavalli, S., Brindha, S., Kaviyarasi, N. S., B. Annadurai, B. and Gangwar, S. K. Antimicrobial activity in leaf extract of neem (*Azadirachta indica* Linn.). *International Journal of Science and Nature*. 2012; 3(1): 110-113.
- [11] Irshad, S., Butt, M. and Younus, H. *In-vitro* antibacterial activity of two medicinal plants neem (*Azadirachta Indica*) and Peppermint. *International Research Journal of Pharmaceuticals*. 2011; 01(01): 9-14.
- [12] Pavithra, P. S., Janani, V. S., Charumathi, K. H., Indumathy, R., Potala, S., and Verma, R. S. Antibacterial activity of plants used in indian herbal medicine. *International Journal of Green Pharmacy*, 2010; 4(1): 22-8.
- [13] Vallis, Sancho. J. and Nacente, Baldris. J. Hand book of microbiological culture media. 9<sup>th</sup> edition, Scharlau Chemie S. A, export@scharlau.com. 2006; pp 68.
- [14] Cheesbrough, M. District laboratory practice in tropical countries, Part 2, Low Price Edition, Cambridge University Press, 2010. pp: 63-70, 137-142, 178-186, 194-197, 689-895.
- [15] Ochai, J. O. and Kolhatkar, A. Medical Laboratory Science and Practice. Tata McGraw-Hill Publishing Limited New Delhi, New York, 2008; pp: 535, 539, 632-635, 788-817.
- [16] Srirangaraj, S., Kali, A. and Charles, P. M. V. A study of antibiogram of *Salmonella enterica serovar Typhi* isolates from Pondicherry India. *Australas Medical Journal*, 2014; 7(4): 185.
- [17] CLSI. Performance Standard for antimicrobial susceptibility testing; twenty-fourth informational supplement; 2015;4(1):1-58.
- [18] Soforowa, E. A. Research on Medicinal Plants and Traditional Medicine in Africa: *Journal of Alternative and Complementary Medicine*, 1996; 2(3): 365-372.
- [19] Trease, G. E. and Evans, W. C. Pharmacognosy. 15<sup>th</sup> Ed. London: Saunders publishers, 2002; Pp. 42-44. 221-229, 246-249, 304-306, 331-332, 391-393.
- [20] Morison, T. R. and Boyd, N. R. Organic chemistry, 6<sup>th</sup> edition, Dorling Kindersley Pvt. Ltd, 2008; pp.626-633.
- [21] Ngan, G. J. Y, Ng, L. M., Lin, R. T. and Teo, J. W. Development of a novel multiplex PCR for the detection and differentiation of *Salmonella enterica* serovars *Typhi* and *Paratyphi A*. *Research in Microbiology*, 2010; 161(4):243-248.
- [22] Alvarez, J., Sota, M., Vivanco, A. V., Pearles, I., Cieterna, R., Rementeria, A. and Garaizar, J. Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. *Journal of Clinical Microbiology*, 2004; 42(4):1734-1738.
- [23] Tanyigna, K. B., Ayeni, J. A., Okeke, E. N., Onah, J. A., Bello, C. S. Antibody levels to *Salmonella typhi* and *paratyphi* in Nigerians. *East African Medicine Journal* 1999; 76(11): 97-100.
- [24] Zailani, S. B., Aboderin, A. O. and Onipede, A. O. Effect of socio-economic status, age and sex on antibody titre profile to *Salmonella typhi/paratyphi* in Ile-Ife, Nigeria. *Nigerian Journal of Medicine*. 2004; 13(4):383-387.
- [25] Ekenze, S. O., Okoro, P. E., Amah, C. C., Ezike, H. A. and Ikefuna, A. N. Typhoid ileal perforation: analysis of morbidity and mortality in 89 children. *Nigerian Journal of Clinical Practices*. 2008; 11(1): 58-62.
- [26] Nasir, A. A., Adeniran, L. O., Abdu-Rahman, L. O., Odi, T. O., and Omotoya, J. A. Typhoid intestinal disease: 32 perforations in 1 patient. *Nigerian Postgraduate Medical Journal*. 2008; 15:55-57.
- [27] Abu, C. C., Oshomole, O. and Adeyemi, S. T. Prevalence of Typhoid fever among out patients visiting IBB specialist hospital and General hospital Minna, Nigeria. *International Research Journal of Science Engineering and Technology*, 2012; 1-14.
- [28] Brandis, H., Link, V., Wursching, F. and Polanetzki, U. Result of phage typing of *Salmonella typhi* and *Salmonella paratyphi B* in the years 1974-1978 from the Federal Republic of Germany including Berlin (West). *Bohlsckentralbl Bacteriol*. 1980; 247(4): 440-459.
- [29] Kam, K. M. Serotype epidemiology and patterns of antibiotic susceptibilities of *Salmonellae* isolated in Hong Kong 1983-1993; *China Medical Journal (Engl)*. 1996; 109(4): 276-281.
- [30] Akinyemi, K. O., Smith, S. I., Oyefolu, A. O., Fasare, K. A. and Coker, A. O. Trends of multiple drug resistance in *Salmonella enterica serovar Typhi* in Lagos, Nigeria. *East and Central African Journal of Surgery*, 2006; 12(1): 83-88.
- [31] Madhulika, U., Harish, B. N. and Parija, C. S. Current pattern in antimicrobial susceptibility of *Salmonella typhi* isolates in Pondicherry. *Indian Journal of Medical Research*. 2003; 120: 111-114.
- [32] De N. and Ifeoma, E. Antimicrobial effects of components of the bark extracts of neem (*Azadirachta indica* A. JUSS). *Technol. Dev*. 2002; 8: 23-26.
- [33] Beg, Amina, Z. and Ahmad, I. Effect of *Plumbago zeylanica* extract and certain curing agents on multi-drug resistant bacteria of clinical origin. *World Journal of Microbiology and Biotechnology*, 2000; 16(8-9):84.