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In vitro Assessment of Selected Antibiotics, Crude Extract of *Dalbergia latifolia* Leaf and Their Combination on MDR *Salmonella enterica* Strain

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Authors' contributions

This work was carried out in collaboration between all authors. Author PAI designed the study, wrote the protocol and supervised the experiment. Authors OTJ and TSA performed the experiment, statistical analysis and wrote the first draft of the manuscript. Authors PAI, OTJ and TSA managed the analyses of the study. Author TSA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the susceptibility pattern *Salmonella enterica* strains to selected antibiotics and extract of *Dalbergia latifolia* as well as their combinatory effect on (multi-drug resistant) MDR salmonella.
Place and Duration of Study: Faculty of Pharmacy, University of Ibadan, Nigeria from October 2012-May 2013.
Methodology: In this study, a total of 11 clinical isolates of *Salmonella enterica* strains were screened *in vitro* against five antibiotics (ampicillin, amoxycillin, chloramphenicol, cotrimoxazole and ceftriaxone) for their antimicrobial susceptibility patterns, and against methanolic extracts of *Dalbergia latifolia* leaves. The isolates were also screened *in vitro* against the combined antibiotics

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and plant extracts using the agar well diffusion method and their MICs determined. Phytochemical screening was done to determine the secondary metabolites present in the plant extracts. *Salmonella enterica* serovar Typhimurium ATCC 14028, a type strain, was used as a reference standard in the identification of the isolates. The isolates were collected across hospitals in South West Nigeria.

Results: All the isolates were multidrug resistance (with each showing resistance to at least two of the antibiotics), with the exception of one susceptible isolate. All the isolates were resistant to chloramphenicol (100%), while the highest susceptible numbers of isolates were observed against ceftriaxone (27.27%), 18.18% were susceptible to each of ampicillin and amoxycillin, while 9.09% were susceptible to cotrimoxazole. MIC values ranged from of 30 µg/mL to >100 µg/mL. All the isolates were susceptible to the extracts of *Dalbergia latifolia* leaves with a zone of diameters equating the CSLI recommendation except for two isolates at a concentration of 25 mg/mL showing lesser activities. The MIC values ranged between 3.125 mg/mL to 75 mg/mL.

Conclusion: The combined antibiotics and plant extracts showed a potentiative and synergistic effects with Fractional Inhibitory Concentration (FIC) values ranging between 0.45-0.75. Among the 11 isolates, 4 isolates (36.36%) showed an additional effect to the combined activities of the antibiotics and plant extracts with a reduced MIC value. From the *in vitro* study, the diameter of the zone of inhibition of the combined halved-MICs (MIC*) of both the plant and antibiotics increased significantly than their individual results. The result of this study showed that the extract of *Dalbergia latifolia* has antimicrobial properties against MDR *Salmonella enterica* strain.

Keywords: MDR; Dalbergia latifolia; Samonella enterica; MIC; FIC.

1. INTRODUCTION

1.1 Epidemiology, Pathophysiology and Immune Responses to Infection

Typhoid fever is a systemic infection caused by common bacterial, transmitted by fecal-oral route following the ingestion of food/water contaminated with the feaces from an infected person [1]. Typhoid or paratyphoid fever, caused by *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi, continues to be endemic in many areas of the developing world, with the highest incidence reported in South Central Asia and South East Asia reaching over 100 per 100 000 cases annually [2]. Gastroenteritis caused by *Salmonella* spp remains a public health concern with the common serotypes from clinical cases across different geographical zones. *Salmonella enterica* serovar Enteritidis is the most common in Europe, Central and South America, while *Salmonella enterica* serovar Typhimurium is predominant in Oceania, North America and Africa [3].

Although precise figures are not available, in 1986 the number of cases of typhoid fever worldwide was estimated at 33 million [4], but according to the most recent estimates, approximately 21 million cases and 222 000 typhoid-related deaths occur annually worldwide [5]. The disease is endemic in many developing countries, particularly in the Indian subcontinent, South and Central America, and Africa, with an estimated average incidence (per 100,000 people per annum) of 150 in South America and 900 in Asia [6]. Travelers to these areas are at risk, and in developed countries of North West Europe, most cases involve returning travellers.

The onset of Typhoid infection begins with ingestion of food or water contaminated with *S. typhi*. Inside the small intestine, the bacteria attach itself to the wall and penetrate through the mucosal layer to the lamina propria. Its translocation through the intestinal lumen by targeting M cells overlying lymphoid tissue. Inside this lymphoid tissue, the Bacteria-induced an influx of macrophages and dendritic cells that ingest the bacteria but fail to destroy them because they have developed a mechanism to resist attack. Thus *Salmonella* remains within macrophages in the lymphoid tissue and migrate into the mesenteric lymph nodes and further invoke an inflammatory response mediated by the release of various cytokines. After a period of 8–14 days of incubation, the clinical symptoms begins with the onset of fever, abdominal discomfort and headache. Naturally, typhoid infection is usually associated with the detection of serum antibodies and mucosal secretory immunoglobulin (Ig) [7,8].

1.2 Treatment

Traditional first-line antibiotic medications include ampicillin, chloramphenicol, and trimethoprim-

sulphamethoxazole. Resistance to these first-line antibiotics defines multidrug resistance in *Salmonella enterica* [9].

The failure of antibiotics therapy for many pathogens has revived the search for antimicrobial compounds from natural sources. Plant extracts have played a lead role in the potential discovery of new antimicrobial agents for the treatment of infectious ailments [10]. Several studies have proposed that natural compounds in combination with antibiotics could offer a new strategy for developing antimicrobial therapies against bacterial infections through potentiating the activity of antibiotics [11].

A variety of plants have been found to possess antimicrobial agents that are useful in treating various infections caused by resistant pathogenic strains of bacteria, an example of such is the use of *Garcinia kola* in combating infection-causing such as *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 9001, *Bacillus cereus.* They were able to show that the bark of the plant has anti-infective properties [12].

Base on the popular report and use as remedies for many infectious diseases, continuous searches for substances with antimicrobial activities in plants are of increase [13]. Plants are rich in a wide variety of secondary metabolites, like tannins, terpenoids, alkaloids, and flavonoids and many others, which have been proved to have *in vitro* antimicrobial properties [14]. The genus *Dalbergia* have been found to have antimicrobial properties against a wide range of Gram-negative *Enterobacteriaceae*. Many plants species have been used in the traditional treatment of infections. These plants are used for the treatment of aphrodisiac, abortifacient, expectorant, anthelmentic, antipyretic, appetizer, allays thirst, vomiting, burning sensation, cures skin diseases, ulcers, diseases of the blood, reduces obesity, used in leucoderma, dyspepsia, dysentery, for diseases of the eye and nose, syphilis, stomach troubles, leprosy, scabies and ringworm [15].

Dalbergia sissoo leaves have been reported to have anti-inflammatory activity, analgesic and antipyretic activities [16]. Mohammad et al. [17], demonstrated that bark extracts of *Dalbergia latifolia* has antioxidant activities against free radicals and thus could serve as a source of primary antioxidant. This study is therefore aimed to validate the antimicrobial susceptibilities of *Salmonella enterica* strains, and to evaluate the antimicrobial activities of the extracts of *Dalbergia latifolia* leaves on the isolates, putting into mind the action of the combined extracts and antibiotics.

2. MATERIALS AND METHODS

2.1 Plant Material

Plant was collect at the botanical garden of the University of Ibadan and was identified as *Dalbergia latifolia* at the herbarium section of the Department of Botany**.**

2.1.1 Method of extraction

The leaf was dusted and air dried at room temperature and then grounded into coarse powder using electric miller (Moulinex). The pulverised leaf was weighted and then subjected to exhaustive Soxhlet extraction with methanol. Extracts were collected and concentrated under reduced pressure using rotary evaporator at 4°C, then reconstituted with 20% dimethyl sulphoxide (DMSO). The stock extracts were kept in the refrigerator at 4°C until use.

2.1.2 Screening method

Presence of various phytochemicals compounds was screen according to Sofowora [18].

Fig. 1. Leaf of *Dalbergia latifolia*

2.2 *Salmonella enterica* Strains

A total of 11 clinical isolates were isolated from hospitals across South West Nigeria and identified based on the standard biochemical characteristics designed for *Salmonella* isolates. Biochemical test such as indole production,

citrate utilization, urease test, Methyl Red-Voges Proskaue (MRVP), Gram's reaction, hydrogen sulphide production was used. Appearance on media such as Bismulth sulphide agar, *Salmonella-Shigella* and MacConkey agar were also used with characteristic circular, shiny surface, small size and slightly raised elevation with a golden yellow pigmentation.

2.2.1 Microorganisms

S4 *Salmonella enterica*
S5 *Salmonella enterica*
S6 *Salmonella enterica*
S7 *Salmonella enterica*
S8 *Salmonella enterica*
S9 *Salmonella enterica*
S11 *Salmonella enterica*
S12 *Salmonella enterica*
S13 *Salmonella enterica*
S16 *Salmonella enterica* serovar Typhi
S24 *Salmonella enterica*

2.3 Antibiotics

Ceftriaxone was purchased from Furen Pharmaceutical Group Company Limited, China. (NAFDAC NO: A4-7210), Ampicillin was purchased from green field pharmaceutical limited, China (NAFDAC NO: 04-7306), chloramphenicol was purchased from Ciron drugs and pharmaceuticals, India (NAFDAC NO: 04-7846), amoxicillin was purchased from Beecham pharmaceuticals, England (NAFDAC NO: 04-0187), cotrimoxazole was purchased from SKG pharm limited, Nigeria. The concentration were prepared in decreasing order from 100 µg/mL to 75 µg/mL, 50 µg/mL and 25 µg/mL. Methanolic extract of crude *Dalbergia latifolia* leaves was dissolved in Dimethyl sulfoxide (DMSO). All process follow standard procedures.

2.4 Antimicrobial Assay

The antimicrobial susceptibility pattern of the isolates was evaluated by the agar well diffusion method, while the Minimum Inhibitory Concentration (MIC) was determined using the broth dilution method. Cells from the overnight cultures were suspended in Mueller Hinton (MH) broth until it produces turbidity equal to the 0.5 McFarland standard No. 1 from which inoculation was done. Antimicrobial activity of the methanolic extracts of *Dalbergia latifolia* leaves was investigated by using the agar well diffusion method as described by Perez et al. [19]. The extract was dissolved in DMSO from which the desired concentrations were prepared and dispensed into the equidistant molten MH agar wells. This was then allowed to diffuse for 1hr before incubation. The zone of inhibition of action was then examined in millimetres. The MIC of the extracts was investigated using the broth dilution method while the Minimum Bactericidal Concentration (MBC) and the Fractional Inhibitory Concentration (FIC) of the extracts were investigated using the agar dilution method. The combination (extract and antibiotics) screening on the isolates was evaluated using the agar-well diffusion method.

3. RESULTS

3.1 Trends of Multiple Drug Resistance to the Antibiotics

Susceptibility pattern to various antibiotics of all the 11 clinical isolates is summarized in Table 1. Among the 11 isolates, 10 (90.91%) isolates demonstrated multidrug resistance (MDR) with each isolates resisting at least 2 or more of the 3 classes of antibiotics (Beta-lactam, Chloramphenicol and Sulphonamides) while 9 (81.82%) isolates demonstrated resistance against both ampicillin and amoxycillin. In addition, 8 (72.73%) isolates were resistant to ceftriaxone, 10 (90.91%) isolates were resistant to cotrimoxazole while all (100%) of the isolates were resistant to chloramphenicol. The MIC values ranged from 30 µg/mL for cotrimoxazole, 50 µg/mL for ceftriaxone, ampicillin and amoxicillin, to >100 µg/mL. The results of the phytochemical screening of *Dalbergia latifolia* leaves showed that tannin, resin, phenol, alkaloids and glycosides are present. The results of the antimicrobial activity of the extracts on the 11 clinical *Salmonella enterica* isolates using the agar well diffusion method is showed in Table 2. At the test concentration, they showed clear wide zones of inhibitions with the exception of 2 isolates at 25 mg/mL showing little activities. The MIC values ranged from 3.125 mg/mL to 75 mg/mL while the MBC values ranged from 12.5 mg/mL to 100 mg/mL Table 3 showed the result of the MIC of the antibiotics. The halved Minimum Inhibitory Concentration of antibiotics and that of the plant extract is shown in Table 3. The MIC* is the combined halved-value of the antibiotics and plant extracts MIC. The results of the Fractional Inhibitory Concentration (MIC*/MIC) of this study are shown in Table 4.

Table 1. Antibiotic susceptibility test (diameter of zone of inhibition in mm)

Isolates	AMP (µg)				AMX (µg)				CEF (µg)				CHL (µg)				COT (µg)			
	30	50	75	100	30	50	75	100	30	50	75	100	30	50	75	100	30	50	75	100
S4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S5	R	R	R	R	R	R	R	R	18	20	23	25	R	R	R	R	R	R	R	R
S6	R	R	R	R	R	R	R	R	31	33	33	35	R	R	R	R	R	R	R	R
S7	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S9	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S11	29	30	33	35	23	25	27	30	R	R	R	R	R	R	R	R	R	R	R	R
S12	32	33	33	35	34	35	37	40	20	23	25	27	R	R	R	R	30	30	31	34
S13	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S24	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

AMX- amoxicillin, AMP- ampicillin, CHL- chloramphenicol, COT- cotrimoxazole, CEF-ceftriaxone, R- resistant.

Table 2. *In vitro* antimicrobial screening of *Dalbergia latifolia diameter of the zone of inhibition (mm) Mean ± SEM.* replicate

Isolates \ Conc.	100mg/mL	75mg/mL	50mg/mL	25mg/mL	MIC mg/mL	DMSO
	Zone of inhibition (mm)					
S4	19±0.2*	19±0.2*	15±0.1*	15±0.3*	75	--
S5	18±0.1*	17±0.2*	16±0.1*	15±0.3*	12.5	--
S6	20±0.2*	19±0.2*	18±0.2*	16±0.1*	12.5	--
S7	23±0.3*	20±0.3*	18±0.2*	--	75	--
S8	22±0.2*	19±0.1*	19±0.2*	15±0.2*	75	--
S9	18±0.1*	18±0.1*	17±0.3*	14±0.2*	75	--
S11	16±0.1*	16±0.1*	14±0.1*	12±0.2*	25	--
S12	28±0.3*	26±0.2*	25±0.1*	22±0.1*	3.125	--
S13	18±0.1*	17±0.2*	17±0.1*	15±0.2*	75	--
S16	16±0.1*	15±0.2*	13±0.2*	11±0.1*	75	--
S24	20±0.2*	18±0.1*	18±0.3*	16±0.3*	12.5	--

**=average results of two*

Table 3. Minimum inhibitory concentration (MIC) of the antibiotics

	Antibiotics (µg/mL)			
	CEF	**AMP**	**AMX**	**COT**
S5	50	--	--	--
S6	50	--	--	--
S11	--	50	50	--
S12	50	50	50	30

CEF- ceftriaxone, AMP- ampicillin, AMX- amoxicillin, COT- cotrimoxazole, S5-S12: isolates

Table 4. Minimum inhibitory concentration of combined antibiotics and plant extract

Isolates	Antibiotic	Pant extract
	CEF (µg/mL)	**mg/mL**
S5	25	6.25
S6	25	6.25
S12	25	1.5625
	COT (µg/mL)	
S12	15	1.5625
	AMP (µg/mL)	
S11	25	12.5
S12	25	1.5625
	AMX (µg/mL)	
S11	25	12.5
S12	25	1.5625

Table 5. Synergic interaction between the tested botanical extract and the antibiotics against the bacterial strains

Isolates	Antibiotics (MIC) (µg/ml)	MIC*	FIC*
	CEF		
S5	50	30.25	0.60
S6	50	31.25	0.62
S12	50	24.56	0.49
	COT		
S12	30	13.56	0.45
	AMP		
S11	50	37.5	0.75
S12	50	22.56	0.45
	AMX		
S11	50	37.5	0.75
S12	50	25.56	0.51

4. DISCUSSION

Infections associated with *Salmonella*e such as Typhoid, Paratyphoid, gastroenteritis, Salmonellosis and the rest are important infections in developing countries, (Nigeria as a case study) and the degree of resistance to antibiotics has been on the increase recently, as evident from the results of this study. The incidence of multidrug resistance, especially to the first lines of antimicrobial therapy against *Salmonella* infections is on the increase. From the results of this study, 90.91% of the isolates are multidrug-resistant while resistance to individual antibiotics, ampicillin (81.82%), amoxycillin (81.82%), ceftriaxone (72.73%), chloramphenicol (100%) and cotrimoxazole (90.91%) is an indication that the prevalence of multidrug resistance is on the increase, especially in South West Nigeria. The results of this study agreed with the work of Ackers et al. [20] wherein a study of 293 people with Typhoid fever, 1% were resistant to nalidixic acid, 17% were resistant to five or more agents including trimethoprim-sulfamethoxazole (cotrimoxazole), ampicillin and chloramphenicol, while 25% were resistant to one or more antibiotics.

Susceptibility pattern to various antibiotics of all the 11 clinical isolates is summarized in Table 1. Among the 11 isolates, 10 (90.91%) isolates demonstrated multidrug resistance (MDR) with each isolates resisting at least 2 or more of the 3 classes of antibiotics (Beta-lactam, Chloramphenicol and Sulphonamides) while 9 (81.82%) isolates demonstrated resistance against both ampicillin and amoxycillin. In addition, 8 (72.73%) isolates were resistant to ceftriaxone, 10 (90.91%) isolates were resistant to cotrimoxazole while all (100%) of the isolates were resistant to chloramphenicol. The MIC values ranged from 30 µg/mL for cotrimoxazole, 50 µg/mL for ceftriaxone, ampicillin and amoxicillin, to >100 µg/mL. The results of the phytochemical screening of *Dalbergia latifolia* leaves showed that tannin, resin, phenol, alkaloids and glycosides are present. The results of the antimicrobial activity of the extracts on the 11 clinical *Salmonella enterica* isolates using the agar well diffusion method is showed in Table 2. At the test concentration, they showed clear wide zones of inhibitions with the exception of 2 isolates at 25 mg/mL showing little activities. The MIC values ranged from 3.125 mg/mL to 75 mg/mL while the MBC values ranged from 12.5 mg/mL to 100 mg/mL Table 3 showed the result of the MIC of the antibiotics. The halved Minimum Inhibitory Concentration of antibiotics and that of the plant extract is shown in Table 3. The MIC* is the combined halved-value of the antibiotics and plant extracts MIC. The results of the Fractional Inhibitory Concentration (MIC*/MIC) of this study are shown in Table 4.

The values obtained for the FIC is evidence that an additive interaction occurs between the

antibiotics and the plant extract since FIC values of below 0.5 should be taking as synergy, 0.5-0.99 is termed indifference while FIC value of 1 is termed antagonistic.

According to Olukoya et al. [21], in most tropical countries, Nigeria inclusive, multidrug resistance *Salmonella typhi* (MDRST) has been on the increase, in which all the clinical isolates he worked with were resistant to trimethoprim-sulfamethoxazole and ampicillin while 50% were resistant to chloramphenicol.

Dated back to the outbreaks of 1989 caused by *Salmonella*, resistant to ampicillin, chloramphenicol, trimethoprim which later extend to streptomycin, sulfonamides, and tetracyclines have been reported in many countries, especially Pakistan [22] and India [23]. MDR strains have also caused outbreaks in Bangladesh [24], several countries in South East Asia [25], and both North and South Africa [26].

Dilruba et al. [27], demonstrated that out of 428 isolates of *Salmonella enterica* serovar Typhi collected, 388 isolates (90.7%) showed resistance to nalidixic acid, 50 isolates (11.7%) had ciprofloxacin zone diameters of >21 mm regarded as susceptible by Clinical and Laboratory Standards Institute (CLSI), 368 (88.3%) had zone diameters of 16 to 20 mm (regarded as intermediate by CLSI), and 10 isolates (2.3%) had zone diameters of 15 mm or less (regarded as resistant by CLSI). On repeat testing, these 10 resistant isolates did not show any zone of inhibition by the disk diffusion method. The phenotypic pattern of resistance showed that 91.4% of the isolates were multidrug resistant. The ciprofloxacin MICs of the 10 resistant isolates were also high and varied between 6.0 and 16.0 µg/ml, thus exceeding the CLSI resistance breakpoint of 4 µg/mL. The levels of resistance to nalidixic acid expressed by all 10 ciprofloxacin-resistant isolates were even higher (≥256 µg/mL), this report supports the results of this study.

Salmonella antibiotic resistance is a global concern that includes multidrug-resistant strains [28]. Overuse, misuse, inappropriate antibiotic prescribing practices, and patient poor compliance lead to a continued increase in multidrug-resistant typhoid fever [29].

According to the results of this study, the minimum inhibitory concentrations (MICs) of the antibiotics were higher compared to the standard concentrations approved for sensitivity on the CLSI table. The MIC values of the amoxicillin, ampicillin, and ceftriaxone were 50 µg/ml while the MIC value for cotrimoxazole was 30 µg/ml. The increased values in the MICs of these antibiotics were evident that resistance to antimicrobial agents by *Salmonella* isolates is on the increase.

Salmonella infections are commonly treated with fluoroquinolones or third-generation cephalosporins, such as ciprofloxacin and ceftriaxone. According to the report of CDC [30], the occurrence of resistance of non-typhoid *Salmonella* isolates to antibiotics was 3.4% for third-generation cephalosporins and 2.6% for quinolones.

Though there is an increase in ciprofloxacin resistance in typhoid and paratyphoid, it is still considered the drug of choice by many physicians. However, in the case of treatment failures, a third-generation cephalosporin and macrolide are good alternatives [31]. The reemergence of chloramphenicol-sensitive strains in prior resistant organisms points towards the concept of antibiotic recycling [32].

Despite the call for a third-generation cephalosporin such as ceftriaxone in the treatment of *Salmonella* infections, the results of this study have shown that resistance even against the ceftriaxone is now emerging and may be attributed to the overuse or inappropriate use of the antibiotics.

5. CONCLUSION

The genus *Dalbergia* have been found to have antimicrobial property against a wide range of Gram-negative *Enterobacteriaceae* but to our knowledge, not much work, if any has been done on *Dalbergia latifolia* against *Salmonella* isolates. The presence of different secondary metabolites in the plant could probably be responsible for the antimicrobial action of the extract on *Salmonella* isolates as evident in the results of the *in vitro* screening of the extract.

The ability of the plant extract to widely inhibit the growth of *Salmonella* isolates is a breakthrough course to serve as new antimicrobial agents from this plant where the active compound should be isolated. The result of this study showed that the extract of *Dalbergia latifolia* has antimicrobial properties. Base on this, it is imperative to say that the extract of the plant could be used in combination with antibiotics to which organism

has developed resistance so as to modify its activities as evident in the results of this study which was in agreement with the finding of Kirtikar and Basu [15], Hussin and El-Sayed [33]. Searching for antibiotic resistance modifying compounds from plants origins are expected to provide the basic knowledge for extraction and purification of leads therapeutically compounds. This could in future be followed by *in vivo* assessments to determine the clinical relevance of such compounds.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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