In vitro study on anti-Salmonella activities of *Boerhaavia diffusa* (L. syn) Leaf extract

Abstract

Various strategies have been employed in the treatment and management of *Salmonella* infection however, *Salmonella* strains have gained resistance to antibiotics. This study was to determine *in vitro* anti-Salmonella activity of *Boerhaavia diffusa* leaf extract against clinical isolate of *Salmonella typhi* and *Salmonella typhi* ATCC 14028. The aqueous and ethanol extracts of *B. diffusa* was studied for their antibacterial activity against pathogenic *Salmonella typhi.* This study was carried out between April and September, 2018. The *in vitro* antibacterial activity was performed by agar well diffusion method and broth dilution using spectrophotometric method and the results were expressed as the average diameter of zone of inhibition of bacterial growth around the well and optical density respectively. It was observed that aqueous extract exerted slightly higher activity than ethanolic extract as revealed by mean diameter of zone of inhibitions at concentration of 200 mg/ml, aqueous extract had 35.21±0.47 mm (*Salmonella typhi* ATCC 14028) compared with ethanol extract 26.41±0.32 mm (clinical). However, in broth dilution method, ethanol extract significantly (p=0.05) reduced the cell, at 48 hours, the optical density of clinical isolate of *S. typhi* treated at concentration of 200 mg/ml of extract was 0.47±0.02 nm while at the same concentration of extract, aqueous extract had optical density of 0.52±0.11 nm respectively. Phytochemical assay revealed that tannin (5.18±0.02 mg/g) and quinone (8.45±0.13 mg/g) in ethanol extract was significantly (p=0.05) higher than aqueous extract while saponin (14.18±0.06 mg/g) was higher in aqueous extract. The ethanol and aqueous extracts of leaves of *B. diffusa* whole plant exhibited significant antibacterial activity against both clinical and typed *Salmonella typhi*. Therefore, the plant extract could be used for the treatment of Salmonellosis, however, the in vivo studies is needed to ascertain the safety of the extract.

Key words: Anti-*Salmonella* activity, plant extracts, agar well diffusion, broth dilution, *Salmonella* strains

Introduction

The bacterium *Salmonella typhi* causes typhoid fever [1, 2]. The bacterium is a gram-negative, motile, non-sporing, non-capsulated bacillus that can be contracted through contaminated water, milk, food or fruits and vegetables or via convalescent or chronic carriers [3]. It has also been linked with zoonotic transmission via reptiles and common domestic pets [4]. *Salmonella enterica*, which is a group of Gram-negative bacterial pathogens capable of infecting humans and animals, cause significant morbidity and mortality worldwide [5]. Certain serotypes adapted to human, such as *Salmonella typhi* (*S. typhi*) and *Salmonella paratyphi* (*S. paratyphi*), usually cause severe diseases in humans, such as enteric fevers (typhoid and paratyphoid fevers). In most endemic areas like Africa, Asia, and Latin America, approximately 90% of enteric fever is typhoid. This disease is an important global health problem with an estimated 16 million cases and 600 000 deaths each year.

Various strategies have been employed in the treatment and management of *Salmonella* infection. Fluoroquinolones and tetracyclines are most commonly used to treat *Salmonella* infections. However, *Salmonella* strains resistant to these antibiotics have been reported in Korea and other countries [6]. One major concern to public health has been the global dissemination of *S.* typhimurium Definitive Type 104, which is resistant to cotrimoxazole, nalidixic acid and ampicilin [7]. The rise in antibiotic-resistant strains has led to increased interest in use of plant materials to develop new effective drugs [6]. Moreover, conventional antityphoid drugs are becoming more and more unavailable to the common man in Africa due to increased cost [8].

The rise in antibiotic-resistant strains has led to increased interest in use of plant materials to develop new effective drugs [6]. It has been reported that 80% of the world population are rural dwellers and rely on medicinal plants for their daily medications, also, plants have been reported to have minimal or no side effects compared to antibiotics [9, 10]. *Boerhaavia diffusa* (Spreading Hogweed in English), belonging to the family of the Nyctaginaceae, is mainly a diffused perennial herbaceous creeping weed of India (known also under its traditional name as *Punarnava*). *Boerhaavia diffusa* is traditionally known in Nigeria as *Etiponla* in Yoruba, *Azeigwe* in Igbo and *Babba-juju* in Hausa. *B. diffusa* is a perennial creeping weed, prostrate or ascending herb, up to 1 m long or more, having spreading branches [11].

The root, leaves, aerial parts and the whole plant of *B. diffusa* (L. syn) are used worldwide for the treatment of a number of disorders e.g. liver complaints, kidney disorders, rheumatism e.t.c.

[12]. The quest to identify and isolate novel phytocompounds from *B. diffusa* has led many researchers to discover various compounds such as flavonoids, alkaloids, glycosides, steroids, triterpenoids, lipids, lignans, carbohydrates, proteins, and glycoproteins from its leaves, stems, seeds and roots [13]. Sourav [14], explored the Anti bacterials from *Boerhaavia diffusa*. In his study, the chloroform and alcohol extracts of the plant were screened against six bacteria viz *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirablis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klebsiella aerogenosa*. Chloroform extract showed activity against *E. coli*, *S. typhimurium* and *P. aeruginosa* while the alcohol extract was active against *P. mirablis* and *S. typhimurium*. The present study was undertaken to further investigate antibacterial activity of *Boerhaavia diffusa* on typed and clinical strains of *Salmonella typhi* with the view to provide scientific evidence for its application as a medicinal plant.

Materials and methods

Collection of leaves of *Boerhaavia*

Fresh leaves of *Boerhaavia diffusa* (Plate 1) were collected from the School of Health Technology, Oda Road, Akure, and identified in the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure Ondo State.

Plate 1: Photograph of *Boerhaavia diffusa* leaf

Preparation of plant extract

Aqueous extraction

The aqueous extractions of the water soluble ingredient were carried out using the filter method. A 2 g of each of the grounded leaves were extracted by successive soaking for 2 days using 50 ml of distilled water in a 250 ml sterile conical flask. The extracts were concentrated in vacuum at 60 ^{o}C and stored in universal bottles and refrigerated at 4 ^{o}C prior to use [15].

Ethanol extraction

The organic solvent leaf extract was prepared by 2 g of plant mixture with ethanol and kept for two days. The extract was concentrated to one-fifth volume, filter sterilized and stored at 4 ^{o}C [15].

Test organism

The clinical bacterial strains were obtained from the Department of Microbiology, Federal University of Technology Akure. Clinical *Salmonella typhi* and typed (ATCC 14028) *Salmonella typhi* were used. The isolates were confirmed based on cultural, morphological and biochemical characteristics following standard methods of identifying *Salmonella typhi* [16]. The bacterial strain was grown in nutrient broth for 12-18 hours at 37 °C on rotary shaker. Cells were grown at 37 °C for 18 hours and cultures were kept at 4 °C.

Antimicrobial susceptibility tests

Standardization of the inoculum

The inoculum was prepared by inoculating colonies of fresh test cultures into sterile distilled water. The turbidity was compared to 0.5McFarland standard prepared according to method of Cheesbrough [16]

Antibacterial susceptibility assay

The extracts were dissolved and diluted using 50 % v/v dimethylsulphoxide (DMSO) to obtain different concentrations (50, 100 and 200 mg) in 1 mL. The 50 mg/ml, 100 mg/ml and 200 mg/ml of the extracts of *B. diffusa* leaves were introduced into the wells of Muller Hinton agar plate. The plates were incubated aerobically at 37 ^{o}C and examined after 24 hours. The plates were examined for microbial growth inhibition and the Inhibition Zone Diameter (IZD) was measured to the nearest millimeter and compared with those produced by the commercial antibiotic ciprofloxacin which was used as control. Effect of extract on anti-*Salmonella* efficacy of the extract in broth was also assayed using spectrophotometric method, the absorbance of the tube was red at 620 nm [16, 17].

Antibiotics sensitivity test using commercial

Antibiotics sensitivity test of the bacterial isolates were determined by disc diffusion method as described by Cheesbrough [16]. Standard inoculum of 18 hours broth was spread on Muller Hinton agar using sterile swab in triplicate. The antibiotic discs were placed on the plate at equidistance. The plates were then incubated for 24 hours at 37°C and diameter of zone of inhibition were measured and recorded. The commercial antibiotics discs (Fondoz Laboratories Ltd, Nigeria) used were; Chloramphenicol (CH) 30 µg, Sparfloxacin (SP) 25 µg, Ciprofloxacin (CPX) 10µg, Amoxicillin (AM) 25µg, Augmentin (AU) 30µg, Gentamycin (CN) 10µg, Pefloxacin (PEF) 5µg, Ofloxacin (OFX) 5µg, Streptomycin (S) 10 µg and Septra (SXT) 30µg.

Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of *Boerhaavia diffusa* Extracts

The Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of the extracts were determined using the broth (tube) dilution technique [18]. Dilutions of the extract in Mueller Hinton broth were prepared in tubes. The concentration of inoculum was also standardized to 0.5 McFarland's turbidity, The Mueller Hinton broth in tubes containing the different concentration of plant extract, 50, 100, and 200 mg/ml were then inoculated with 0.5 ml of the standardized culture. The tubes were then incubated at 37 °C for 24 hours. MIC and MBC values were recorded.

Screening of phytochemical compounds

The various solvent extracts of the powder of leaves of *Boerhaavia diffusa* were subjected to phytochemical tests for the identification of various action constituents using the method of Marcelin *et al.* [17]. The following major pharmaceutical valuable phytochemical compounds were analyzed qualitatively and quantitatively; alkaloids, phenols, tannins, flavonoids, quinones, saponins, terpenoids, sterols and cardiac glycosides.

Statistical analysis of data

Data obtained were subjected to analysis of variance and means were compared using Duncan's New Multiple Range Test (DNMRT) with the aid of SPSS software at $p \leq 0.05$ level of significance.

Results and Discussion

Salmonellosis and enteric fever are always a public health concern in most developing countries, which are mostly low or middle-income countries with inadequate sanitation and hygiene, particularly, regarding food, water and disposal of human excreta [17]. Different plants and their parts (flowers, buds, leaves, stem, bark, fruits, skin, pulp and root) have been used for thousands of years to enhance the flavour and aroma of food. In addition, plants are rich in a wide variety of second metabolites such as Alkaloids, Flavonoids, Phenols, which were found in vitro to have antimicrobial properties [17, 19].

In this study, extracts of *Boerhaavia diffusa* leaves were investigated for antibacterial activity against *Salmonella typhi*. Plant extracts were used to investigate antibacterial activity against two bacterial strains (Clinical *Salmonella typhi* and *Salmonella typhi* ATCC 14028). In this study, antibacterial activity of *B. diffusa* leaf extracts was compared against the test bacteria with activities of model antibiotics.

The test organisms used for this study were identified based on biochemical characteristics common to *Salmonella typhi*. The result is presented in Table 1. The antibiotic sensitivity patterns of commercial antibiotics on the two strains of *S. typhi* are presented in Figure 1. The result revealed that the zones of inhibition of antibiotics against typed isolates was higher than that of clinical isolates however, chloramphenicol had highest inhibition against the isolates (STC=24.30±0.42 mm, STT=24.36±0.07 mm). The higher antibacterial activity of model antibiotics is not surprising, since the antibiotics are in a refined state. The standard antibiotics (ampicillin, amoxycillin, ciprofloxacin, ofloxacin, chloramphenicol) used in this study are first line drugs employed in the treatment of typhoid fever [1].

The results of antibacterial activity of both water and ethanol crude extracts of *B. diffusa* showed anti*Samonella* activity on the two strains of *S. typhi* tested at different concentrations, with aqueous extract exerting slightly higher activity than ethanolic extract as revealed by mean diameter of zone of inhibitions, 200 mg/ml of aqueous extract had highest (35.21±0.47) zone of inhibition (Figure 2). Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of the extracts is shown in Tables 2. The ethanol and aqueous extract had the same MIC (100 mg/ml) on typed isolate, also, there was no difference in the MIC and MBC of ethanol and aqueous extract on typed isolate. The aqueous and ethanol extracts exhibited different zones of inhibition against the isolates, however, aqueous extract had higher zones of inhibition than ethanol extract. Antimicrobial action may be due to the synergistic action of different chemical constituents, some of which probably are last upon extraction with solvent [15, 17, 20]. Water could be a better extraction solvent than ethanol for *B. diffusa* leaf, also, the demonstration of higher activity by the aqueous solvent may be an indication that the phytoconstituents in the plant leaves are more soluble in water than the organic solvent [21]. The antimicrobial potential of *B. diffusa* and other plants sourced from traditional healers through an ethnobotanical survey of anti-infective plants in Egbado South in Ogun State, Nigeria was previously reported by Abo and Ashidi [22]. This study also corroborates the findings of Madani and Jain [23] who reported higher anti-*Salmonella* activity in aqueous extract of *Terminalia belerica* than chloroform and acetone extracts. It has been reported that different phytoconstituents have different degrees of solubility in different types of solvents depending on their polarity. In a traditional setting, water is the solvent largely used to prepare these concoctions.

The anti-*Salmonella* efficacy of *Boerhaavia diffusa* extracts in broth was assayed and were shown in Figure 3, 4, 5 and 6. The result presented in Figure 3 revealed the effect of ethanol extract on clinical isolate of *S. typhi,* it was noted that the extract significantly ($p<0.05$) reduced the cell, at 48 hours, the optical density of clinical isolate of *S. typhi* treated with 50, 100, 200 mg/ml of extract were 0.52±0.03, 0.50±0.10, 0.47±0.02 nm respectively while at the same concentration of extract, aqueous extract had optical density of 0.64±0.21, 0.54±0.03, 0.52±0.11 nm respectively (Figure 4). Also, the anti-*Salmonella* efficacy of *B. diffusa* ethanol extracts on typed isolate of *S. typhi* is shown in Figure 5. It was observed that the extract significantly ($p<0.05$) reduced the cell, at 48 hours, the optical density were of typed isolate of *S. typhi* treated with 50, 100, 200 mg/ml of extract were 0.49±0.00, 0.48±0.01 and 0.37±0.12 while at the same concentration, aqueous extract had optical density of 0.62±0.03, 0.53±0.11 and 0.49±0.21 nm respectively (Figure 6). It was noted from this study that plant extracts tested by microdilution technique and the optical density was measured after 48 hours showed that ethanol extract had higher anti-*Salmonella* activity compared to aqueous extract which was higher in values obtained from agar well diffusion technique. It could be that the bioactive components in ethanol extract did not diffuse into agar in agar well but was able to inhibit microbial cells directly in broth. This was previously reported by other findings that the active components of the extract does not diffuse into Muller Hinton agar, however, they were able to cause inhibition of microbial cells in broth microdillution [6, 24].

Both plant extracts (ethanolic and aqueous) were subjected to preliminary qualitative phytochemical evaluation. The phytochemical profiles of the two solvent extracts from plant used in this study are presented in Table 2. The analysis revealed the presence of alkaloids, phenol, glycosides, steroids, carboxylic acid, reducing sugar, flavonoids, saponins, tannins, proteins, triterpenoids, quinines, carbohydrates and sterols. Also, tannin (5.18±0.02 mg/g) and quinone (8.45±0.13 mg/g) in ethanol extract was significantly ($p<0.05$) higher than aqueous extract while saponin (14.18±0.06 mg/g) was higher in aqueous extract. The preliminary qualitative phytochemical screening carried out showed that the leaf extracts of *B. diffusa* contain vital secondary metabolites such as alkaloids, saponins, tannins and glycosides. The bioactive compounds in medicinal plants have been reported to be the active principles responsible for the pharmacological potentials of medicinal plants [25]. The presence of these chemicals in the leaves and root of these plants justifies the local uses of these plants for the treatment of various ill conditions. Phytoconstituents such as saponins, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections [6, 26]. Ethanol extract of *Boerhavia diffusa* leaves possess some phytochemicals like Alkaloids, Anthraquine, Glycoside, Flavanoids and Tannins. Saponins are natural glycosides that act as hypoglycemic, antifungal and serum cholesterol lowering agents in animals [27]. Saponins are essential elements in ensuring hormonal balance and synthesis of sex hormones [28]. Tannins are bitter polyphenolic compounds that hasten the healing of wounds. They also possess anti-diuretic and anti-diarrhea properties [28]. Terpenoids was present in both ethanolic extract of and aqueous extracts of AOU and AFU. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer. Terpenoids are also known to possess antimicrobial, antifungal, anti- parasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory and immunemodulatory properties [29]. The presence of these compounds promises its potential application in the treatment of microbial ailment. However, tannins were present in aqueous extract of but not in the ethanolic extract. Saponin and flavanoid is higher in the aqueous extract of the leaf (14.18 and 11.26 mg/g) than the ethanolic extract (6.36 and 9.98 mg/g).

Table 1: Biochemical characteristics of *Salmonella* strains

Biochemical characteristics	*Salmonella typhi* (Clinical isolate)	*Salmonella typhi* (ATCC 14028)
Gram reaction	-ve	-ve
Shape	Rod	Rod
Motility	+ve	+ve
Catalase	+ve	+ve
Coagulase	-ve	-ve

Citrate	+ve	+ve
H_2S	+ve	+ve
Lactose	-ve	-ve
Glucose	+ve	+ve
Fructose	+ve	+ve
Sucrose	-ve	-ve
Galactose	+ve	+ve
Indole	-ve	-ve
Methyl red	+ve	+ve
Voges-Proskaeur	-ve	-ve
Oxidase	-ve	-ve

Key: -ve = negative +ve = positive

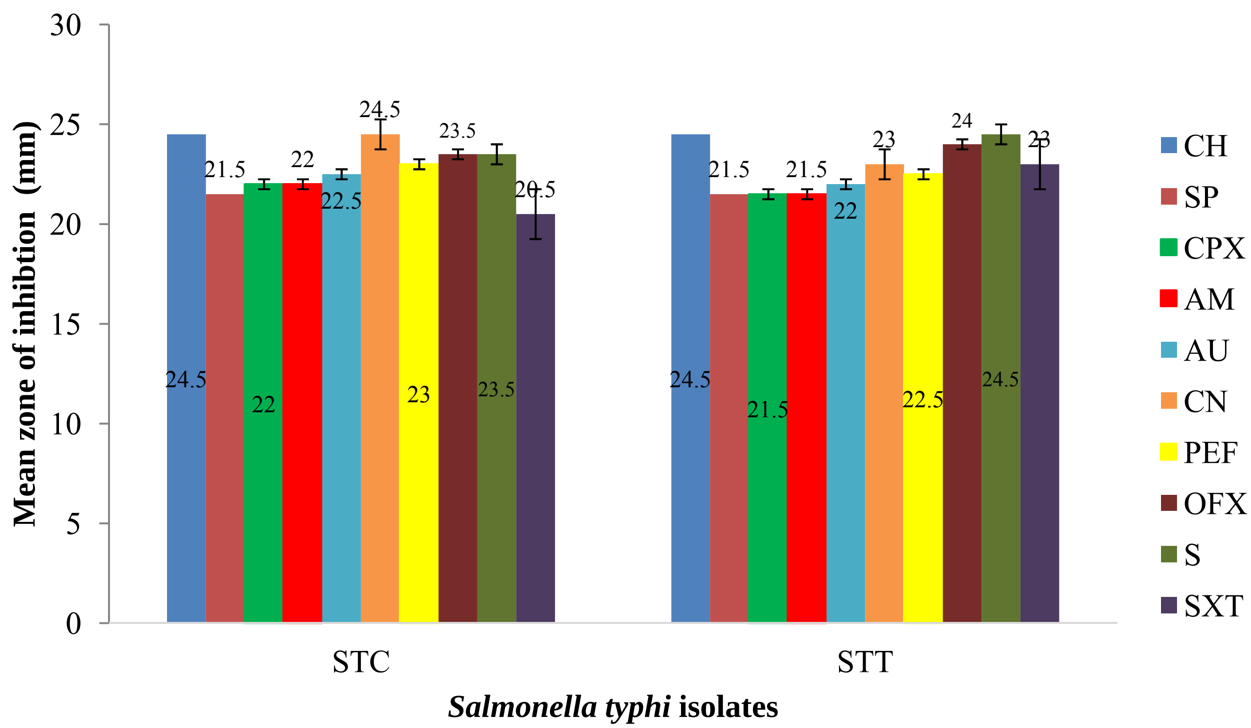

Figure 1: Antibiotic sensitivity pattern of commercial antibiotic discs on *S. typhi* strains

Key: STC – *Salmonella typhi* (clinical isolate), STT – *Salmonella typhi* (typed isolate), Chloramphenicol (CH) 30 µg, Sparfloxacin (SP) 25 µg, Ciprofloxacin (CPX) 10µg, Amoxicillin (AM) 25µg, Augmentin (AU) 30µg, Gentamycin (CN) 10µg, Pefloxacin (PEF) 5µg, Ofloxacin (OFX) 5µg, Streptomycin (S) 10 µg and Septra (SXT) 30µg.

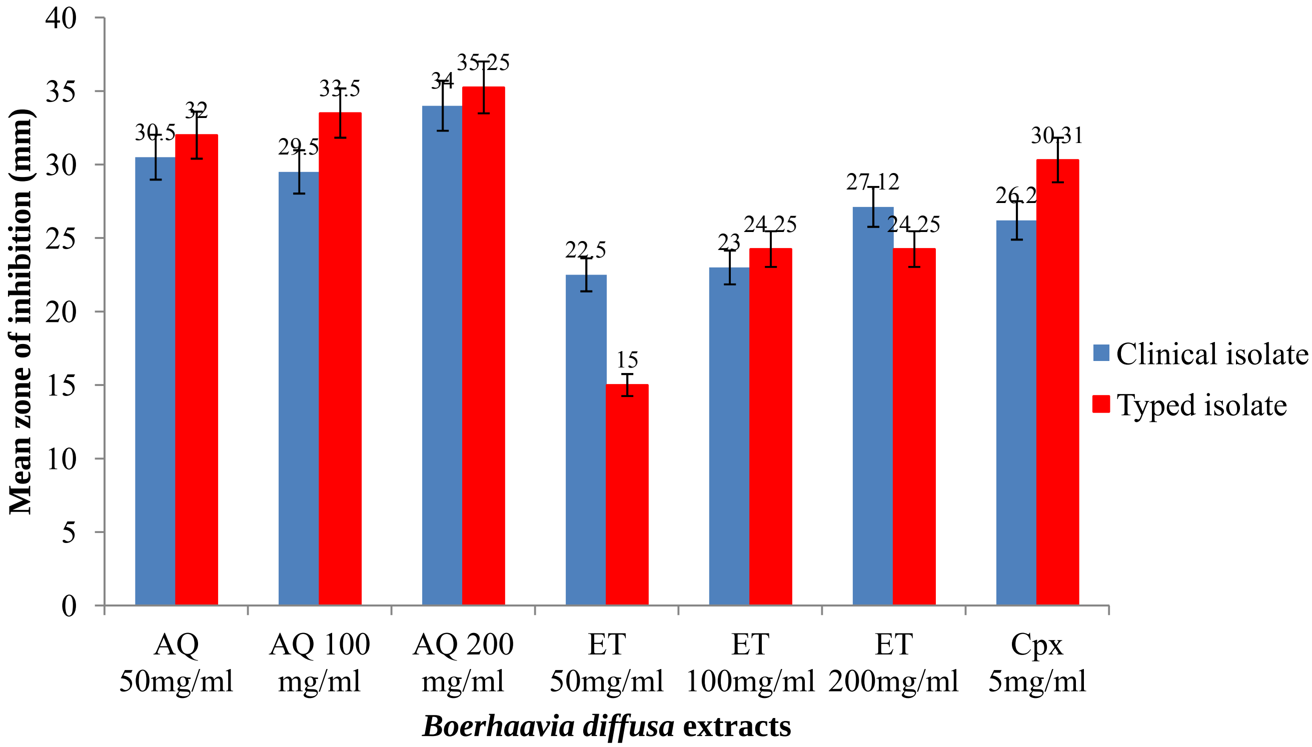

Figure 2: Anti*Salmonella* activity of *Boerhaavia diffusa* extracts

Key: AQ = Aqueous extracts of *Boerhaavia diffusa*, ET= Ethanolic extracts of *Boerhaavia diffusa*, Cpx= Ciprofloxacin

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of *Boerhaavia diffusa* extracts on *Salmonella*

***Boerhaavia diffusa* extracts**	**Ethanolic extract**		**Aqueous extract**	
	S1	**S2**	**S1**	**S2**
MIC (mg/ml)	100	100	50	100
MBC (mg/ml)	50	100	50	100

Key: S1 - *S. typhi* (Clinical isolate), S2 – *S. typhi* (ATCC 14028)

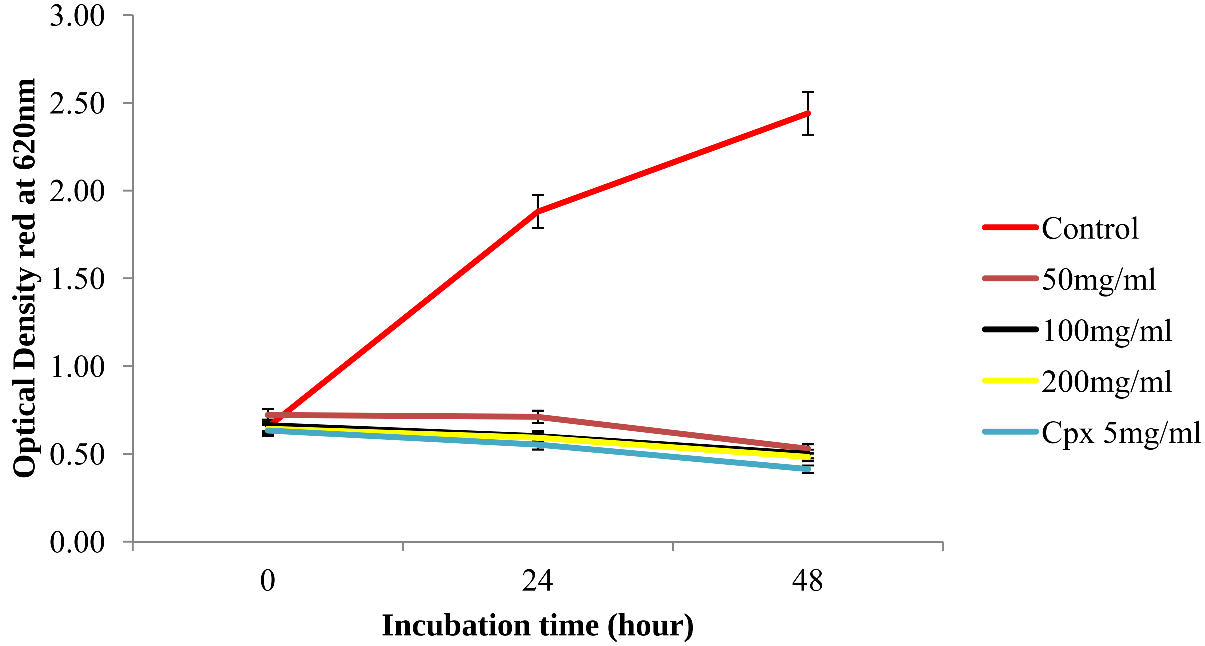

Figure 3: Effect of *Boerhaavia diffusa* ethanol extract on Clinical isolate

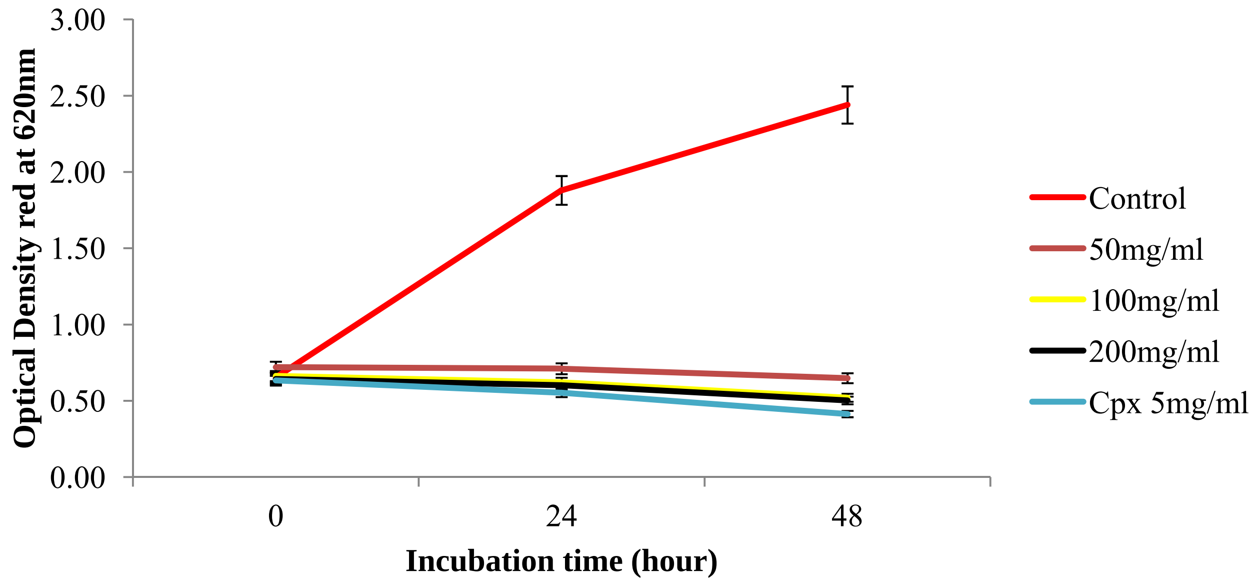

Figure 4: Effect of *Boerhaavia diffusa* aqueous extract on clinical isolate

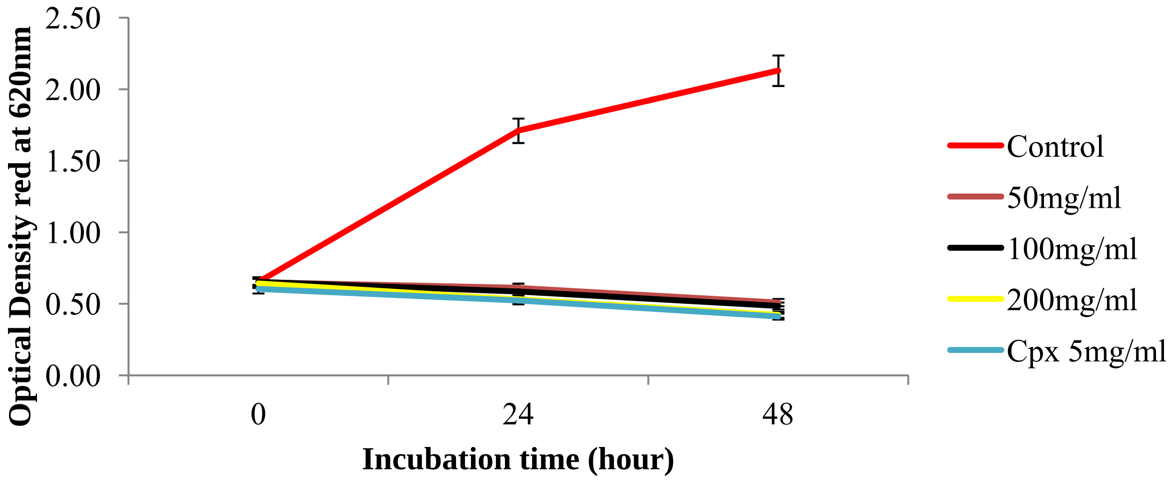

Figure 5: Effect of *Boerhaavia diffusa* ethanol extract on typed isolate

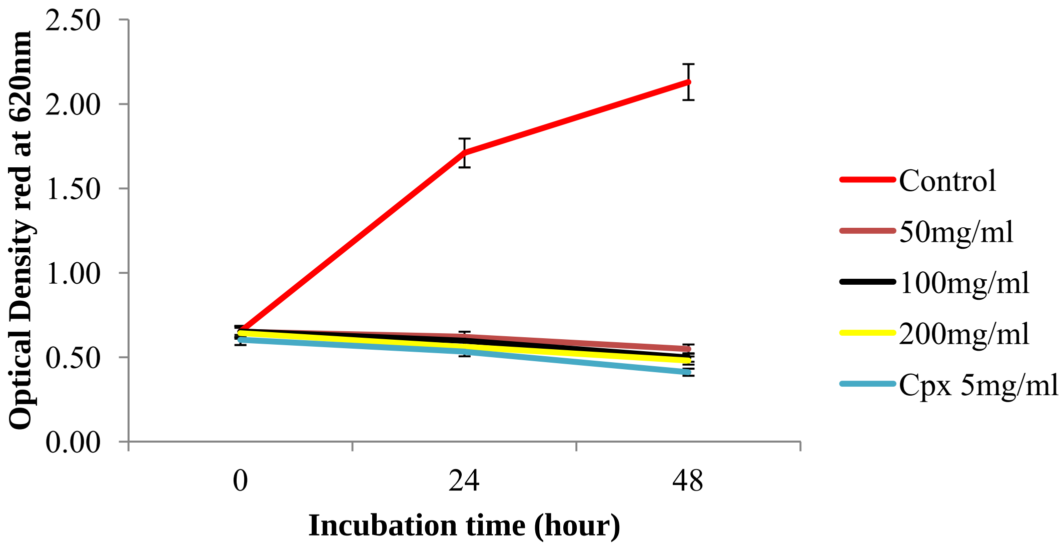

Figure 6: Effect of *Boerhaavia diffusa* aqueous extract on typed isolate

Table 3: Qualitative analysis of phytochemicals in *Boerhavia diffusa* leaf extracts

Phytochemical	Ethanolic extract	Aqueous extract
Alkaloids	-	+
Tannins	-	+
Flavonoids	+	+

Quinones	+	_
Saponins	+	+
Terpenoids	+	-
Sterols	+	-
Cardiac Glycosides	-	+
Phenols	+	+

Key : + = Present, - = Absent

Table 4: Quantitative phytochemical screening of aqueous and ethanol extracts of *B. diffusa*

Phytochemicals	Ethanolic extract	Aqueous extract
Tannins (mg/g)	5.18±0.02^{a}	3.90±0.22^{a}
Quinones (mg/g)	8.45±0.13^{b}	6.60±0.31^{a}
Saponins (mg/g)	6.36±0.24^{a}	14.18±0.06^{b}
Triterpenoids (mg/g)	8.56±0.08^{a}	8.89±0.31^{a}
Steroid (mg/g)	9.03±0.11^{a}	6.73±0.14^{a}
Glycosides (mg/g)	30.39±0.06^{b}	28.29± 0.03^{a}
Flavonoids (mg/g)	9.98±0.61^{a}	11.26±0.33^{a}

Conclusion

Most of the antibiotics used nowadays have lost their effectiveness due to development of resistant genes in microbes. The antibiotics are sometimes associated with side effects such as hypersensitivity, immune suppression and allergic reaction.

More interest is being shown in developing alternative antimicrobial drugs for the treatment of infectious diseases without side effects. The results of our present study demonstrates anti-*Salmonella* activity of aqueous and ethanol extract of *Boerhavia diffusa*, tannin and quinone were higher in ethanol extract while saponin was higher in aqueous extract, using agar well diffusion, the aqueous extract showed higher anti-*Salmonella* efficacy while the broth microdillution examined by spectrophotometer revealed that ethanol extract had higher anti-*Salmonella* efficacy. In the present study, the anti-salmonella activity of *Boerhaavia diffusa* may be attributed to individual or synergistic effect of phytoconsitituents present in it. The ethanol and aqueous extracts of leaves of *B. diffusa* whole plant exhibited significant antibacterial activity against both clinical and typed *Salmonella typhi*. Therefore, the plant extract could be used for the treatment of Salmonellosis, however, the in vivo studies is needed to ascertain the safety of the extract.

Recommendation

Based on our findings, it is therefore recommended that both agar well diffusion and broth dilution method should be used to affirm the antimicrobial efficacy of the plant extracts.

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