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Research Article

Synergistic Effect of Combined Antibiotic and Methanolic Extracts of *Withania somnifera* and *Catharanthus roseus* against MDR *Salmonella enterica* Serovar Typhi

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Typhoid has become the most neglected and majorly affecting disease of tropical and subtropical countries, including India, and is among the most important global health problems, as the emergence of multidrug resistance has shut down the effect of antimicrobial agents and makes it difficult to control the bacteria inside the host. Hence, there is a great need to develop some natural-based drugs, which will be new promising natural therapeutic interventions with high efficacy and lesser side effects in comparison to synthetic drugs already available for typhoid treatment. The present study completely focused on the synergistic effect of bioactive constituents of medicinal plants in combination to synthetic drugs for enhancing the bacterial eradication mechanism. The methanolic extracts of 25 medicinal plants were screened for their antisalmonella effect and out of which only 2 plants were studied further on the basis of their high effectiveness against 17 MDR isolates using well diffusion assay and MIC/MBC determination. The synergistic activity was assessed with two nonantibiotics (ibuprofen and paracetamol) and 3 antibiotics (ceftazidime chloramphenicol, and trimethoprim) using methods of growth inhibitory indices (GIIs) and fractional inhibitory concentration index (FICI). The highly effective methanolic extracts were of *W. somnifera* and *C. roseus*, and the synergism was obtained in terms of GIIs and FICI values of 0.9 and 0.3 and 0.9 and 0.5, respectively, fulfilling the criteria for both extracts, respectively. The results for combinations of plant extracts and antibiotics ceftazidime, trimethoprim, and chloramphenicol and nonantibiotics (analgesic drugs) ibuprofen and paracetamol showed good synergistic activity (100%, 88%, 50%, 45%, and 35%, respectively) against the MDR isolates of *S. Typhi*. The present study also suggests that in the future, combined treatment with the antibiotics and the bioactive compounds can enhance the immune system to perform better action against the external pathogens as well as in the treatment of internal pathogens, and the combinations will be the successful immune modulators.

1. Introduction

Salmonella Typhi is an intracellular pathogen that causes typhoid fever in humans (the only natural hosts and reservoir of infection) [1]. This disease has become a major public health problem in developing countries [2]. The emergence of MDR strains of *S. Typhi* has encouraged urgency to develop more effective typhoid vaccines [3]. Despite recent advances in the area of vaccinology [4, 5], the pace of progress is not fast enough and needs to be accelerated [6]. Hence, there is a need of continuous surveillance and sharing of resistant data for *Salmonella* among countries worldwide [7] to ensure the effectiveness of control programs [8].

The global emergence of multidrug-resistant typhoid bacilli has become the most threatening issue and has limited the effectiveness of current drugs, causing explorative treatment failures [9]. The containment of this drug resistance requires thrust for the development of new potent antimicrobial compounds as alternatives to existing antibiotics [10]. However, the development of new antimicrobial drugs is not encouraging with only a few new drugs being licensed in the last few years [11, 12]. This mismatch between the slow development of new drugs and the fast emergence of resistant strains makes the disease management miserable. As an alternative or perhaps a sustainable option and attempts to improve the efficacy of available

antibiotics, particularly the older and cheaper drugs have been suggested [13].

Medicinal plants as an alternative continue to play an important role in the healthcare systems from ancient times, particularly in the developing countries, where the herbal medicine has a longer and uninterrupted history of use [14]. The medicinal plants are the target for new therapeutic interventions due to the production of a wide variety of secondary metabolites, many of which have been reported to be of therapeutic value. This raises the prospects of obtaining novel chemotherapeutic compounds if these vastly untapped resources could be adequately explored. Indeed, the plants might be a source of biocompounds that may potentiate the activity of antibiotics against resistant pathogens. These compounds may be useful as resistance-modifying, modulating, or reversal agents. While the routine practice just screens the plant extracts for direct antimicrobial compounds, searching of resistance-modifying compounds from natural resources may also improve the efficacy of antibiotics when used in combination. These compounds must be more effective and attractive as they allow the recycling of old and relatively cheaper antibiotics that have been rendered ineffective due to resistance. Several studies have proposed that plant-derived compounds in combination with antibiotics are the novel methods for developing therapies against bacterial infections as they may enhance the effect of antibiotics in combination due to their synergistic effects [15–17]. Hence, the present study was also focused on the use of medicinal plants in combination with the synthetic drugs as alternative method of treatment against the emergence of MDR *S. Typhi* strains.

2. Material and Methods

2.1. Ethical Justification. The project was approved by institute's ethical committee wide IEC no. SUIEC/15/04 (attached certificate in supplementary data).

2.2. Collection, Maintenance of Clinical Isolates, and Screening of MDR Phenotype. A total of 40 clinical isolates of *S. Typhi* processed from the confirmed patient's blood and studied for their cultural characteristics and 1 positive control MTCC-733 strain obtained from IMTECH, Chandigarh, were taken for the study. All the isolates were inoculated in glycerol stocks and transported to the MIPL laboratory, Shoolini University (Solan). The isolates were further confirmed based on selective culture characterization, staining, and biochemical identifications. The blood samples from which the isolates were obtained were also studied for WIDAL (Table-2 supplementary data) and ELISA using specific serotyped antigens.

2.3. Antibiotic Susceptibility Assay. All the isolates were screened to know the MDR phenotype by disc diffusion assay [18] which was performed with 12 different groups of antibiotics. The complete growth inhibition around each of the discs was measured by using a transparent plastic ruler. Zone diameters of inhibition were compared with the standards as given by HiMedia (Zone Scale). The percent inhibition of diameter growth was calculated in the following manners:

$$\%\text{inhibition} = \frac{\text{diameter of sample} - \text{diameter of control} \times 100}{\text{diameter of control}}. \quad (1)$$

The minimum inhibitory concentration against *S. Typhi* was determined by broth microdilution reference method (CLSI M7-A7) after the determination of the antimicrobial activity by agar well diffusion method. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms, and for quantitative analysis, OD was taken at 595 nm.

$$\%\text{inhibition} = \frac{(\text{OD of control} - \text{OD of media control}) - (\text{OD of test} - \text{OD of extract control} \times 100)}{\text{OD of negative control} - \text{OD of media control}}. \quad (2)$$

2.4. Collection of Plant Material. A total of 25 medicinal plants were collected from Distt. Solan (HP) area (medicinal plants used in the study enlisted in Table-1 of supplementary data). The plants were authenticated from the Faculty of Basic and Environmental Sciences, Shoolini University wide Herbarium nos. SUBMS/BOT-S203 and SUBMS/BOT-S272. As per their antityphoid efficacy and phytochemical-based analysis, only two plants, i.e., *Withania somnifera* and *Catharanthus roseus*, were further studied for synergistic activity against the MDR strains.

2.5. Study of Combined Effect of Extracts and Antibiotics [19]

2.5.1. GII (Growth Inhibitory Index) Method. Antibacterial activity was measured using well diffusion method according to National Committee for Clinical Laboratory Standard 2000. Presence of turbidity was adjusted according to 0.5 McFarland standards, and the Mueller-Hinton agar plates were prepared.

The growth inhibitory indices (GIIs) were calculated for the well diffusion method as ZDI in combination/ZDIs of both agents in combination [20], to corroborate the synergistic activity (in the forms of ZDI) of the extraction combination with the antibiotics and nonantibiotics (analgesic drugs) as control. The effect was said to be synergistic if the value of GIIs > 0.5, additive if GIIs = 0.5, or antagonistic effect was measured in terms of GIIs < 0.5 [21].

2.5.2. FIC (Fractional Inhibitory Concentration) Method: Efficacy of Plant Extracts in Lowering the MIC of Antibiotics. Firstly, MIC of antibiotics and plant extract was determined separately. To determine the combined effect of antibiotic and plant extract, combinations of different concentrations ranging from 1/2 × MIC to 8 × MIC of each were used. This assay was performed in 96-well ELISA plate. By this assay, a fixed concentration of active compound was determined which decreased the MIC of the antibiotic. The following formula was used for the determination of FICI:

$$\text{FIC index} = \frac{\text{MIC of antibiotic in combination}}{\text{MIC of antibiotic alone}} + \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}}. \quad (3)$$

Combinations were classified as follows: synergistic as $\sum FIC \leq 0.5$, additive as $0.5 < \sum FIC < 1$, indifferent as $1 \leq \sum FIC \leq 2$, and antagonistic as $\sum FIC > 2$ ([21–23] and Salaria and [23]).

2.5.3. Bactericidal Kinetic Assay. Bactericidal kinetic assays were performed by the method of Gadhi et al. [24] against *S. Typhi* with minor modifications. A series of tubes containing nutrient broth and sterile extracts at varying concentrations (5-50 mg/ml) and both positive (antibiotic) and negative (broth culture of *S. Typhi*) controls was inoculated with 10^5 CFU of *S. Typhi* and incubated at 37°C. After 0, 1, 2, 4, 6, 8, 10, and 12 h of incubation, bacterial inoculums from each tube were plated on triple sugar iron agar. Plates were incubated overnight at 37°C, and numbers of viable bacteria were counted by colony counter.

2.5.4. Fractionation of Crude Plant Extracts. The methanolic leaf extracts (ME) were fractionated by solvent–solvent partitioning to obtain five water (WtF), ethyl acetate (EaF), chloroform (CfF), n-butanol (BtE), and n-hexane (HxF) fractions [25] (further isolated by column chromatography).

2.5.5. Synergistic Assay of Active Fractions with Antibiotic and Nonantibiotics. The combined effect of active fractions was studied in combination to antibiotics and nonantibiotics as described in GII and FICI analysis methods.

2.5.6. FTIR Analysis of Active Fraction and Bioassay-Guided Fractions. The Agilent Cary 630 Series FTIR spectrometer (Agilent Technologies) was used to study functional group present in chloroform fraction and their subtractions. The main purpose of this study was to identify the presence of certain functional groups in a molecule and the unique collections of the absorption bands to confirm the identity of a pure compound. The frequencies and intensities of the infrared bonds provide information about the nature of the molecular IR spectroscopy [26].

2.5.7. Statistical Analysis. The obtained results were analyzed statistically, and values were represented as mean ± SD. Statistical analysis of collected data was also conducted using CRD three factorial analysis carried out on three factors. The least significant difference at 5 percent level was used for the analysis of significant data among treatments [27].

3. Results

3.1. Isolation, Characterization, and Identification of Salmonella Typhi Isolates. A total no. of 40 suspected typhoid bacillus samples were cultured on the blood agar, incubated at 37°C for 24 h, and then examined macroscopically and microscopically. The isolates were grown on general purpose media (nutrient agar), differential media (MacConkey agar), and selective media (bismuth sulphite agar and XLD). Primarily, *S. Typhi* was identified based on colony characteristics and further subjected to microscopic and biochemical identifications. The growth was observed based on colony characteristics produced by *S. Typhi* on various culture media. It produces dome or disc-shaped colonies on nutrient agar, nonlactose fermenting colorless colonies on MacConkey agar, black-colored colonies on bismuth sulphite agar, and pink- or black-centered colonies on XLD agar. The Gram-stained smear was examined under the microscopic oil immersion lens that revealed Gram-negative rod-shaped bacilli. The isolates were further characterized by biochemical tests. The isolates were found to be catalase positive, indole nonfermenting, methyl red positive, and negative for the VP test. Furthermore, the bacilli were found to be noncitrate utilizing and nonurease degrading but nitrate reducing. The bacilli ferment glucose, mannitol, maltose, and sucrose by producing acid but were nonlactose fermenting. The bacilli were found to be highly motile as they had spread-type growth on the semisolid medium. The typhoid bacilli had high agglutination titer in WIDAL and ELISA (profile available as supplementary data Table-2).

3.2. Screening of MDR Strains of Salmonella Typhi. All the 40 *S. Typhi* isolates were processed for ASA (antimicrobial susceptibility assay) using disc diffusion method. It was found that out of 40 strains, 42% (17/40) were MDR (antibiogram pattern available as supplementary data Table-3 and Table-4). The results were interpreted as resistant, intermediate, and sensitive as per the CLSI guidelines, 2012.

Antibiogram profile of the resistant isolates against the antimicrobials was studied (description given but the data not shown here). Antibiotic sensitivity assay was carried out for *S. Typhi* isolates ($n = 40$) using more than 20 antibiotic discs (HiMedia, Mumbai) belonging to different classes; out of the screened isolates, only 42% (17/40) were found MDR (supplementary data Table-3 and Table-4). The isolates were found to be highly resistant to penicillin and vancomycin (62.5%) followed by ofloxacin and tetracycline (47.5%); kanamycin and ampicillin (45%); trimethoprim, sulfanilamide, and cotrimoxazole (42.5%); and amikacin (40%), while amoxicillin showed 39.5% and clindamycin and chloramphenicol 35%; low resistance was found against ciprofloxacin (10%). In addition, the isolates were found to be 99% resistant to azithromycin and gentamicin, while 100% sensitive for ceftazidime, levofloxacin, and cefotaxime.

The minimum inhibitory concentration (MIC) of MDR *S. Typhi* isolates ($n = 17$) was determined. The MIC values $\geq 16\,\mu$l/ml were considered as borderline (BL) resistant, whereas the increase in MIC values was designated as highly resistant and all the isolates were found with MIC values above this considered MDR. It was found that none of the isolates showed inhibition at the MIC conc. of 0.5-8.0 μl/ml. The isolates found beyond the MIC conc. of 256 μl/ml were highly resistant towards the antibiotics studied. The predominant MIC concentration was found to be ≥256 μl/ml for *S. Typhi* isolates against the resistant antibiotics followed by 128 μl/ml (Table-4 in supplementary data).

3.3. Antityphoid/Antisalmonella Assay of Plant Extracts. The antibacterial activity of methanolic extracts (yield enlisted in Table-5 supplementary data) was performed on 17 MDR *S. Typhi* isolates. A total of 25 methanolic extracts obtained from different parts of traditionally used medicinal plants were evaluated for their antisalmonella activity. Out of these

25 methanolic extracts (MEs), the MDR isolates were found to be highly sensitive towards 10/25 plants: *D. purpurea* ($n = 16$), *C. roseus* ($n = 14$), *R. serpentine* ($n = 12$), *W. somnifera* ($n = 18$), *G. glabra* ($n = 10$), *C. sinensis* ($n = 15$), *T. chebula* ($n = 15$), *J. regia* ($n = 18$), *C. sativa* ($n = 18$), and *P. granatum* ($n = 13$), although the MDR isolates showed intermediate sensitivity towards 6/25 medicinal plants: *C. citratus* ($n = 7$), *N. jatamansi* ($n = 5$), *M. officinalis* ($n = 13$), *F. vulgare* ($n = 16$), *A. paniculata* ($n = 13$), and *S. cumini* ($n = 18$). In addition to this, we have observed that the MDR isolates were found resistant to 9/25 medicinal plants: *C. longa* ($n = 4$), *C. pseudolimon* ($n = 18$), *B. suaveolens* ($n = 18$), *C. annum* ($n = 18$), *A. nilotica* ($n = 13$), *O. vulgare* ($n = 5$), *F. religiosa* ($n = 8$), *A. racemosus* ($n = 9$), and *O. tenuiflorum* ($n = 6$). Among the methanolic extracts of medicinal plants which showed sensitivity towards the MDR *S. Typhi* isolates, the predominant plant extract was found to be of *W. somnifera*, *J. regia*, and *C. sativa* followed by *C. sinensis*, *T. chebula*, *C. roseus*, *D. purpurea*, *R. serpentine*, *P. granatum*, and *G. glabra*.

3.4. Minimum Inhibitory Concentration and Minimum Bactericidal Count (MIC/MBC in mg/ml) of the Most Effective Methanolic Extracts. The most effective plant extracts found in the zone diameter of inhibition (ZDI) pattern were processed for their MIC and MBC determination. The results of MIC and MBC revealed that the methanolic extract of *W. somnifera* found to have the lowest MIC and MBC values (conc. 0.156 mg/ml-0.625 mg/ml) followed by *C. roseus* (conc. 0.156 mg/ml-1.25 mg/ml), *C. sativa* (conc. 0.156 mg/ml-2.5 mg/ml), *J. regia* (conc. 0.156 mg/ml-2.50 mg/ml), *R. serpentine* (conc. 0.312 mg/ml-2.50 mg/ml), *T. chebula* (conc. 0.312 mg/ml-2.50 mg/ml), and *D. purpurea* (conc. 0.312 mg/ml-5.0 mg/ml), and the less effective MIC and MBC were found for *P. granatum* (0.625 mg/ml-5.0 mg/ml) against the MDR isolates studied. The results conclude that the highly active plants were *W. somnifera*, *C. roseus*, *C. sativa*, *J. regia*, *R. serpentine*, *T. chebula*, and *D. purpurea*, and the least effective was *P. granatum*.

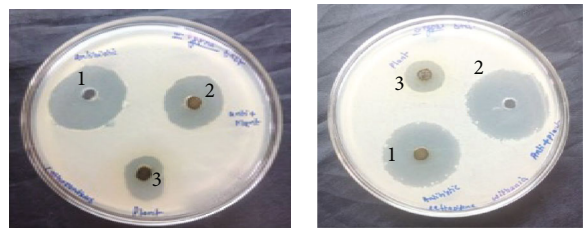
3.5. Synergistic Assay of Medicinal Plants with or without Antibiotics and Nonantibiotics. Methanolic extracts of medicinal plants ($n = 8$) were evaluated for synergistic activity with 3 antibiotics (trimethoprim, chloramphenicol, and ceftazidime) and two nonantibiotics (paracetamol and ibuprofen) against MDR *S. Typhi* isolates ($n = 17$). The methanolic extracts of *W. somnifera* and *C. roseus* were found most synergistic based on GII (growth inhibitory index) and FICI (fractional inhibitory concentration index) values (0.9 and 0.3 and 0.9 and 0.5, respectively) (Table-6 supplementary data).

3.6. Synergistic Effect Using Growth Inhibitory Indices (GIIs) and Fractional Inhibitory Concentration Indices (FICI). The methanolic extracts from the plants showed synergistic effect (*W. somnifera* and *C. roseus*) and were further studied to determine the synergism, antagonism, and indifferent activity between the extracts and antibiotics/nonantibiotics. The zone of inhibition (for GIIs) and MIC (for FICI) were determined separately and then in combination (Figure 1 and Tables 1–4). The results revealed that the methanolic plant extracts in combination with antibiotics chloramphenicol (50%), ceftazidime (100%), and trimethoprim (88%) and nonantibiotics paracetamol (35%) and ibuprofen (45%) showed potent synergistic activity against the MDR isolates of *S. Typhi*. The synergistic effect was found to be effective on more strains by the GII method (56.9%) than the FICI method (36.4%) (Tables 1–4). Furthermore, the results also revealed that the synergistic effect was found higher in combination to antibiotics (100%) than the nonantibiotics (61%) (Tables 1–4). Hence, the findings suggest that the methanolic extracts of *W. somnifera* and *C. roseus* contain bioactive compounds which have properties to enhance the effect of antimicrobials on the MDR *S. Typhi* isolates or are potent enhancers of the effectivity to decrease the resistance among the *S. Typhi* isolates.

3.7. Bactericidal Kinetic Assay of Methanolic Extracts of W. somnifera and C. roseus against MDR S. Typhi Isolates. The bactericidal kinetic assay was performed for highly effective/synergistic plants: *W. somnifera* and *C. roseus*. Four different concentrations (12.5 mg/ml, 25 mg/ml, 50 mg/ml, and 100 mg/ml) of methanolic extracts were studied for the time-dependent inhibition of the MDR strains (1.5×10^8 CFU/ml). The results of time-kill kinetic study of the methanolic extract against *S. Typhi* showed 99.9% reduction at 50 mg/ml concentration. The maximum reduction of 2 $\log_{10}$ at 50 mg/ml was achieved after 12 h by the methanolic extracts of *W. somnifera* and *C. roseus* for controlling the microbes. Approximately 98% reduction in bacterial growth was observed after 24 hours of incubation (Figures 2(a) and 2(b)). It was found from the present study that plants showed bacteriostatic activity up to 6-8 h and after 8 h. The plant extracts completely inhibit the growth of MDR strains of *S. Typhi* (Figures 2(a) and 2(b)).

3.8. Antisalmonella Assay of Fractions against MDR S. Typhi Isolates. The fractions were obtained using the separating funnel method and further studied for antisalmonella activity on 17 MDR *S. Typhi* isolates. Only 57% (4/7) plant fractions were found active, of which the chloroform fractions from both plants, *W. somnifera* and *C. roseus*, were found to be highly sensitive (ZDIs 20 ± 0.3 and 15 ± 2, respectively) against MDR isolates followed by ethyl acetate, butanol, and aqueous extracts, respectively (Figure 1 and Table-7, 8 supplementary data).

3.9. Synergistic Effect of Fractions of W. somnifera and C. roseus against MDR S. Typhi Isolates. The ethyl acetate, chloroform, butanol, and aqueous fractions of *W. somnifera* and *C. roseus* were evaluated for synergistic activity with three antibiotics, i.e., trimethoprim, chloramphenicol, and ceftazidime, and two nonantibiotics, i.e., paracetamol and ibuprofen, against 17 MDR *S. Typhi* isolates. The chloroform fractions of *W. somnifera* and *C. roseus* were found most synergistic based on growth inhibitory index (GII, 0.7) and fractional inhibitory concentration (FIC, 0.5) values. The



(a) (b)

Figure 1: Representation of the synergistic effect of medicinal plants in combination to the antimicrobial agents: (a) synergistic activity of *C. roseus* in combination with chloramphenicol and (b) synergistic activity of *W. somnifera* in combination with ceftazidime (1 = antibiotic alone; 2 = antibiotic+plant extract; 3 = plant extract alone).

Table 1: Synergistic effect of *W. somnifera* in combination with antibiotics and nonantibiotics on MDR strains of *S. Typhi* (GII method).

Strains	Synergistic effect with antibiotics						Synergistic effect with nonantibiotics			
	GIIs (TR)	Activity (TR)	GIIs (C)	Activity (C)	GIIs (CAZ)	Activity (CAZ)	GIIs (P)	Activity (P)	GIIs (IBU)	Activity (IBU)
ST	0.7	Synergistic	0.7	Synergistic	0.6	Synergistic	0.4	Antagonistic	0.4	Antagonistic
NS-3	0.6	Synergistic	0.7	Synergistic	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic
NS-4	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.6	Synergistic	0.4	Antagonistic
NS-5	0.5	Additive	0.5	Additive	0.6	Synergistic	0.5	Additive	0.6	Synergistic
NS-6	0.6	Synergistic	0.7	Synergistic	0.7	Synergistic	0.5	Additive	0.4	Antagonistic
NS-7	0.9	Synergistic	0.6	Synergistic	0.7	Synergistic	0.5	Additive	0.5	Additive
NS-9	0.8	Synergistic	0.4	Antagonistic	0.7	Synergistic	0.3	Antagonistic	0.6	Synergistic
NS-10	0.8	Synergistic	0.5	Additive	0.6	Synergistic	0.4	Antagonistic	0.3	Antagonistic
NS-11	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.4	Antagonistic	0.3	Antagonistic
NS-12	0.6	Synergistic	0.5	Additive	0.6	Synergistic	0.4	Antagonistic	0.0	Antagonistic
NS-13	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic
NS-14	0.0	Antagonistic	0.4	Antagonistic	0.6	Synergistic	0.5	Additive	0.6	Synergistic
NS-15	0.8	Synergistic	0.5	Additive	0.7	Synergistic	0.6	Synergistic	0.6	Synergistic
NS-16	0.8	Synergistic	0.7	Synergistic	0.6	Synergistic	0.4	Antagonistic	0.3	Antagonistic
NS-17	0.7	Synergistic	0.5	Additive	0.6	Synergistic	0.4	Antagonistic	0.3	Antagonistic
NS-23	0.7	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.3	Antagonistic	0.4	Antagonistic
NS-34	0.9	Synergistic	0.6	Synergistic	0.7	Synergistic	0.4	Antagonistic	0.4	Antagonistic
NS-40	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.3	Antagonistic	0.4	Antagonistic

ME = methanolic extracts; TR = trimethoprim; C = chloramphenicol; CAZ = ceftazidime; P = paracetamol; IBU = ibuprofen; GIIs = growth inhibitory indices.

GII values of chloroform fraction were observed as 0.7 and 0.9, higher than the normal value and FIC value of 0.5, respectively, indicating the synergistic effect followed by the ethyl acetate fraction where the GII value was 0.6 and FIC was 0.9, whereas the effect of aqueous fraction was found antagonistic as it gives GII values 0.45 and FIC 2.0. However, the effect of butanol fraction was found to be additive since here the GIIs and FIC were 0.5 and 1.0, respectively (Table 5).

3.10. FTIR Analysis of Chloroform Fractions of W. somnifera and C. roseus for the Detection of Functional Groups. The chloroform fractions were found to be most effective and synergistic towards the MDR isolates of *S. Typhi*. Hence, the chloroform fractions of both plants, *W. somnifera* and *C. roseus*, were studied for FTIR analysis. The FTIR study showed the differences between the organization and positioning of functional groups of various bioactive compounds present in chloroform fractions of both plants (Figures 3(a) and 3(b) and Table 6).

4. Discussion

Typhoid fever is known to be a major public health problem in tropical and subtropical countries including India [28]. The emergence of multidrug resistance strains of *Salmonella* against existing antibiotics is going to cause serious problems, and spread of drug-resistant strains throughout the endemic regions is an alarming feature. Hence, there is a need to discover and develop reliable and cost-effective drugs to overcome the problem of multidrug resistance. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as an alternative treatment for infectious diseases.

Table 2: Synergistic effect of *C. roseus* in combination with antibiotics and nonantibiotics on MDR strains of *S. Typhi* (GII method).

Strains	Synergistic effect with antibiotics						Synergistic effect with nonantibiotics			
	GIIs (TR)	Activity (TR)	GIIs (C)	Activity (C)	GIIs (CAZ)	Activity (CAZ)	GIIs (P)	Activity (P)	GIIs (IBU)	Activity (IBU)
ST	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.4	Antagonistic	0.5	Additive
NS-3	0.7	Synergistic	0.5	Additive	0.6	Synergistic	0.0	Antagonistic	0.4	Antagonistic
NS-4	0.8	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.7	Synergistic	0.8	Synergistic
NS-5	0.8	Synergistic	0.8	Synergistic	0.7	Synergistic	0.5	Additive	0.5	Additive
NS-6	0.8	Synergistic	0.8	Synergistic	0.7	Synergistic	0.6	Synergistic	0.6	Synergistic
NS-7	0.9	Synergistic	0.8	Synergistic	0.7	Synergistic	0.6	Synergistic	1.0	Synergistic
NS-9	0.7	Synergistic	0.7	Synergistic	0.7	Synergistic	0.7	Synergistic	0.7	Synergistic
NS-10	0.9	Synergistic	0.5	Additive	0.7	Synergistic	0.5	Additive	1.0	Synergistic
NS-11	0.5	Additive	0.4	Antagonistic	0.6	Synergistic	0.4	Antagonistic	0.3	Antagonistic
NS-12	0.5	Additive	0.5	Additive	0.6	Synergistic	0.4	Antagonistic	0.4	Antagonistic
NS-13	0.6	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.4	Antagonistic	0.4	Antagonistic
NS-14	0.7	Synergistic	0.5	Additive	0.6	Synergistic	0.3	Antagonistic	0.3	Antagonistic
NS-15	0.6	Synergistic	0.5	Additive	0.6	Synergistic	0.5	Additive	0.8	Synergistic
NS-16	0.6	Synergistic	0.5	Additive	0.8	Synergistic	0.4	Antagonistic	0.6	Synergistic
NS-17	0.7	Synergistic	0.4	Antagonistic	0.6	Synergistic	0.7	Synergistic	0.6	Synergistic
NS-23	0.7	Synergistic	0.5	Additive	0.6	Synergistic	0.8	Synergistic	0.7	Synergistic
NS-34	0.8	Synergistic	0.5	Additive	0.7	Synergistic	0.8	Synergistic	0.8	Synergistic
NS-40	0.8	Synergistic	0.5	Additive	0.7	Synergistic	0.5	Additive	0.5	Additive

ME = methanolic extracts; TR = trimethoprim; C = chloramphenicol; CAZ = ceftazidime; P = paracetamol; IBU = ibuprofen; FICI = fractional inhibitory concentration index.

Table 3: Synergistic effect of *W. somnifera* in combination with antibiotics and nonantibiotics on MDR strains of *S. Typhi* (FICI method).

S. no.	Strains	Synergistic effect with antibiotics						Synergistic effect with nonantibiotics			
		FICI (C)	Activity (C)	FICI (CAZ)	Activity (CAZ)	FICI (TR)	Activity (TR)	FICI (P)	Activity (P)	FICI (IBU)	Activity (IBU)
1	ST	1	Indifferent	0.4	Synergistic	1.2	Indifferent	1.2	Indifferent	1.5	Indifferent
2	NS-3	1.25	Indifferent	0.5	Synergistic	1.2	Indifferent	1.5	Indifferent	1.2	Indifferent
3	NS-4	1	Indifferent	1	Indifferent	0.5	Synergistic	0.5	Synergistic	1.2	Indifferent
4	NS-5	1.5	Indifferent	1.2	Indifferent	1.5	Indifferent	1.2	Indifferent	1.5	Indifferent
5	NS-6	1.5	Indifferent	0.6	Synergistic	1.2	Indifferent	1.1	Indifferent	1.2	Indifferent
6	NS-7	0.7	Additive	0.4	Synergistic	0.5	Synergistic	1.2	Indifferent	1.5	Indifferent
7	NS-9	0.5	Synergistic	0.5	Synergistic	1.1	Indifferent	0.7	Additive	1.2	Indifferent
8	NS-10	0.3	Synergistic	0.7	Additive	0.7	Additive	1.1	Indifferent	1.2	Indifferent
9	NS-11	0.4	Synergistic	1.5	Indifferent	0.7	Additive	1.2	Indifferent	1.2	Indifferent
10	NS-12	1.5	Indifferent	1.5	Indifferent	0.5	Synergistic	1.2	Indifferent	0.6	Additive
11	NS-13	1	Additive	1	Indifferent	0.5	Synergistic	1.5	Indifferent	1.5	Indifferent
12	NS-14	1.5	Indifferent	0.4	Synergistic	1.5	Indifferent	1.5	Indifferent	1.5	Indifferent
13	NS-15	0.7	Additive	0.4	Synergistic	1.0	Indifferent	1.5	Indifferent	1.5	Indifferent
14	NS-16	0.5	Synergistic	1	Indifferent	0.3	Synergistic	1.2	Indifferent	1.2	Indifferent
15	NS-17	0.7	Synergistic	0.7	Additive	1.1	Indifferent	1.2	Indifferent	1.2	Indifferent
16	NS-23	0.4	Synergistic	0.4	Synergistic	1.2	Indifferent	1.1	Indifferent	0.5	Synergistic
17	NS-34	0.7	Additive	0.6	Additive	1.1	Indifferent	0.5	Synergistic	1.1	Indifferent
18	NS-40	1.5	Indifferent	1	Indifferent	2.1	Antagonistic	1.1	Indifferent	0.7	Additive

ME = methanolic extracts; TR = trimethoprim; C = chloramphenicol; CAZ = ceftazidime; P = paracetamol; IBU = ibuprofen; FICI = fractional inhibitory concentration index.

Table 4: Synergistic effect of *C. roseus* in combination with antibiotics and nonantibiotics on MDR strains of *S. Typhi* (FICI method).

S. no.	Strains	Synergistic effect with antibiotics						Synergistic effect with nonantibiotics			
		FICI (C)	Activity (C)	FICI (CAZ)	Activity (CAZ)	FICI (TR)	Activity (TR)	FICI (P)	Activity (P)	FICI (IBU)	Activity (IBU)
1	ST	0.5	Synergistic	0.4	Synergistic	0.7	Additive	0.7	Additive	1	Indifferent
2	NS-3	0.4	Synergistic	0.7	Additive	0.5	Synergistic	1.5	Indifferent	1.5	Indifferent
3	NS-4	1	Indifferent	0.4	Synergistic	0.7	Additive	1.5	Indifferent	1	Indifferent
4	NS-5	1.2	Indifferent	1	Indifferent	1.5	Indifferent	1.5	Indifferent	1.5	Indifferent
5	NS-6	1.2	Indifferent	0.7	Additive	1.5	Indifferent	1.5	Indifferent	1.5	Indifferent
6	NS-7	0.7	Additive	0.3	Synergistic	1	Indifferent	0.5	Synergistic	1	Indifferent
7	NS-9	1.2	Indifferent	1	Indifferent	1.1	Indifferent	1.1	Indifferent	1.2	Indifferent
8	NS-10	1	Indifferent	0.4	Synergistic	0.5	Synergistic	1	Indifferent	0.7	Additive
9	NS-11	1.2	Indifferent	0.7	Additive	1.2	Indifferent	1.2	Indifferent	1.5	Indifferent
10	NS-12	0.4	Synergistic	1	Indifferent	0.4	Synergistic	1.5	Indifferent	0.6	Additive
11	NS-13	0.5	Synergistic	1.5	Indifferent	1.1	Indifferent	0.4	Synergistic	0.7	Additive
12	NS-14	1	Indifferent	1	Indifferent	2.1	Antagonistic	1.1	Indifferent	1.1	Indifferent
13	NS-15	1	Indifferent	0.5	Synergistic	2.5	Antagonistic	0.7	Additive	1	Indifferent
14	NS-16	0.6	Additive	0.7	Additive	0.5	Synergistic	0.5	Synergistic	0.5	Synergistic
15	NS-17	1	Indifferent	1	Indifferent	0.6	Additive	0.6	Additive	0.6	Additive
16	NS-23	0.7	Additive	1	Indifferent	1.2	Indifferent	1.1	Indifferent	0.3	Synergistic
17	NS-34	0.4	Synergistic	0.7	Additive	1	Indifferent	0.6	Additive	0.6	Additive
18	NS-40	1	Indifferent	0.5	Synergistic	1.2	Indifferent	1	Indifferent	1.1	Indifferent

ME = methanolic extracts; TR = trimethoprim; C = chloramphenicol; CAZ = ceftazidime; P = paracetamol; IBU = ibuprofen; FICI = fractional inhibitory concentration index.

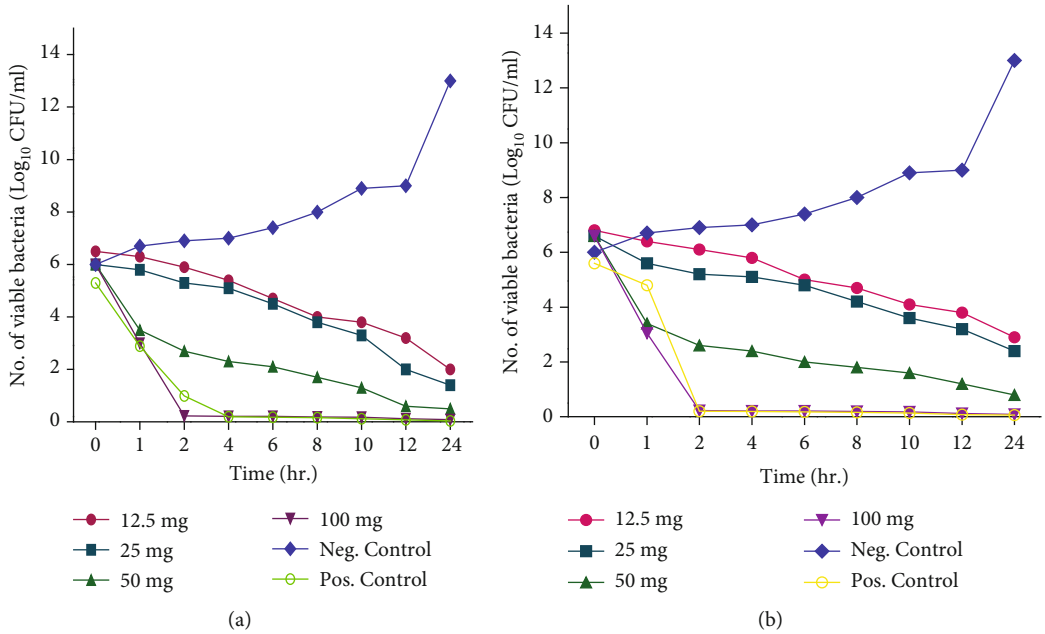


Figure 2: Kinetics of bacterial inhibition assay using methanolic extracts of leaves of (a) *W. somnifera* and (b) *C. roseus*.

Synergism is said to be a phenomenon in which two different compounds combine in such manners so that they can enhance their individual activity or effectiveness. The medicinal plants were screened in a systematic manner to obtain the most synergistic plants in combination to antibiotics and nonantibiotics. A total of 25 medicinal plants were obtained for the study based on their ancient uses in the treatment of fever and as a booster of the immune system.

Table 5: Synergistic assay of various fractions of methanolic extract of *W. somnifera* and *C. roseus* against *S. Typhi* isolates.

S. no.	Fractions	GIIs	Effects	FICs	Effects
1	Ethyl acetate	0.6	Synergistic	0.8	Additive
2	Chloroform*	0.7	Synergistic	0.5	Synergistic
3	Butanol	0.5	Additive	1	Antagonistic
4	Aqueous	0.45	Antagonistic	2	Antagonistic

GIIs = growth inhibitory indices; FIC = fractional inhibitory concentrations.

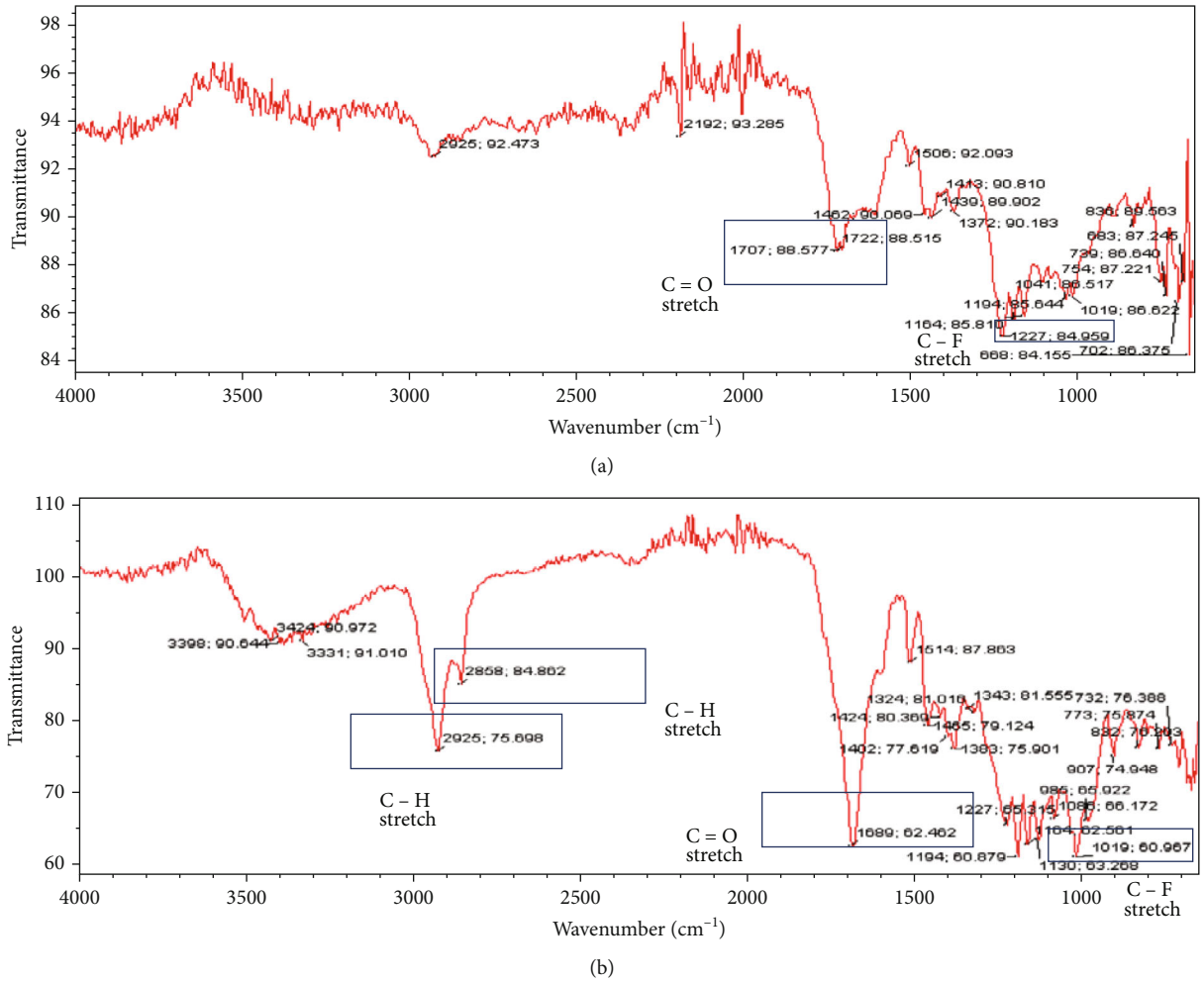


Figure 3: FTIR spectra of chloroform fractions obtained from the medicinal plants. (a) *C. roseus* and (b) *W. somnifera*.

The different parts of the plants selected for the study were dried, and the extract yield was obtained in the range of 100 mg to 2.0 g from the 10 g powder.

In the present study, the antibacterial activity of methanolic extracts was studied on 17 MDR *S. Typhi* isolates. A total of 25 methanolic extracts of medicinal plants were evaluated for their antisalmonella activity. Out of these 25 methanolic extracts (MEs), 10 plants, i.e., *D. purpurea*, *C. roseus*, *R. serpentine*, *W. somnifera*, *G. glabra*, *C. sinensis*, *T. chebula*, *J. regia*, *C. sativa*, and *P. granatum*, were found to be highly sensitive against MDR isolates of *S. Typhi*. In support, Arora et al. [29] have suggested that the methanolic and hexane extracts of medicinal plants are much more effective against the bacterial strains by studying the antimicrobial efficacy of methanolic and hexane extracts against *S. Typhimurium* and *E. coli*.

The phytochemical analysis also revealed the presence of all secondary metabolites studied in *W. somnifera*. Similar findings were revealed by Akinpelu et al. [30] that alkaloid and flavonoids present in the plant parts contribute to their biological effectiveness. They further stated that the most common biological properties of alkaloids and flavonoids are antimicrobial efficacy and toxicity against cells of foreign organisms.

The methanolic extracts analyzed for synergistic effect (*W. somnifera* and *C. roseus*) were further studied to

Table 6: FTIR analysis of chloroform fractions of *C. roseus* and *W. somnifera*.

	Wavenumber (cm^{-1})	Functional groups
C. roseus	1707	C=O stretch (aldehydes)
	1227	C-F stretch (alkyl halides)
W. somnifera	2925	C-H stretch (alkanes and alkynes)
	2858	C-H stretch (alkanes and alkynes)
	1689	C=O stretch (carboxylic acid)
	1019	C-F stretch (alkyl halides)

determine the synergism, antagonism, and indifferent activity between the extracts and antibiotics/nonantibiotics against the MDR strains on individual basis. The zone of inhibition (for GIIs) and MIC (for FIC) were observed and calculated separately as well as in combination. The results revealed that the methanolic plant extracts in combination with antibiotics chloramphenicol (50%), ceftazidime (100%), and trimethoprim (88%) and nonantibiotics paracetamol (35%) and ibuprofen (45%) showed potent synergistic activity against the MDR isolates of *S. Typhi*. The synergistic effect was found to be effective on more strains by GII method (56.9%) than the FIC method (36.4%). Furthermore, the results also revealed that the synergistic effect was found high in combination to antibiotics (100%) than the nonantibiotics (61%). Hence, the findings suggested that the methanolic extracts of *W. somnifera* and *C. roseus* contain bioactive compounds which have properties to enhance the effect of antimicrobials on the MDR *S. Typhi* isolates or are potent enhancers of the efficacy to decrease the resistance among the *S. Typhi* isolates. The *C. roseus* has been observed with potent bioactivity, including anticancer, anti-inflammatory, antimicrobial, and antidiabetic activity in several studies [31–33], thus justifying its use as ethnomedical plant. *C. roseus* have majority of alkaloids, while besides alkaloids, it also produces other compounds, including anthocyanins, flavonoids, iridoids, and steroids [33, 34].

Accordingly, Muhammad et al. [35] reported the antibacterial potential of crude extracts of different parts (viz., leaves, stem, root, and flower) of *C. roseus* against clinically important bacteria. Similarly, Zhang et al. [36] reported the antibacterial potential of the root extract of *W. somnifera* against *S. Typhi*. The leaves of the *W. somnifera* (Indian chemotype) contain 5 unidentified alkaloids, 12 withanolides, several free amino acids glycosides, chlorogenic acid, condensed tannins, flavonoids, and glucose [37]. Several studies have been reported that the alkaloid withaferin A is able to inhibit the growth of various Gram-positive bacteria, acid-fast bacteria, aerobic bacilli, and the pathogenic fungi. The studies also suggested the active effect of alkaloids on *Micrococcus pyogenes varaureus* and partial inhibition of *Bacillus subtilis* glucose-6-phosphatedehydrogenase. Withaferin A also reported in the similar studies to inhibit the growth of Ranikhet virus, and the shrub extract was found to inhibit Vaccinia virus and *Entamoeba histolytica* [37–39]. In the present study, the inhibition was observed to be significantly very high against *S. Typhi* and other strains used in this study which concordance to the previous studies.

The bactericidal kinetic assay was performed for highly effective/synergistic plants: *W. somnifera* and *C. roseus*. The present study revealed that the time-kill kinetic assay of methanolic extract against MDR *S. Typhi* showed significantly very high (99.9%) reduction with 50 mg/ml concentration. The maximum reduction of 2 $\log_{10}$ at 50 mg/ml was analyzed after 12 h by the methanolic extracts of *W. somnifera* and *C. roseus* for controlling the microbes. Approximately 98% reduction in bacterial growth was observed after 24 h of incubation. It was found from the present study that plants showed bacteriostatic activity up to 6 h-8 h, and after 8 h, the plant extracts completely inhibit the growth of MDR strains of *S. Typhi*. Furthermore, the fractions of methanolic extract have also been studied to know the sensitivity of a particular fraction for the isolation and extraction of bioactive compound. The present study revealed that the chloroform fraction of methanolic extract of *W. somnifera* and *C. roseus* was observed highly sensitive against the MDR isolates of *S. Typhi* followed by ethyl acetate, butanol, and aqueous extracts, respectively, while the other fractions were not found active against the MDR isolates. The chloroform fractions were observed to be the most effective and synergistic towards the MDR isolates of *S. Typhi*. Hence, the chloroform fractions of both plants, *W. somnifera* and *C. roseus*, were further analyzed by physical properties and FTIR. The color of the fractions was found to be black and mist green along with 265°C (*C. roseus*) and 276-278°C (*W. somnifera*) melting temperature, respectively. The fractions have solubility with methanol, chloroform, and DMSO. The FTIR analysis revealed the differences between the positioning and organization of functional groups of various bioactive compounds present in chloroform fractions of both plants.

Similarly, a study conducted on similar group of microbes with medicinal plant extracts showed stimulated effect on the activity of antibiotics to inhibit the cytoplasmic targets [40, 41], while a similar study conducted by Baddley and Poppas on the fungal strains revealed the synergy of broader spectrum activity between plant extracts and antibiotics and a decreased risk of emergence of resistant strains [42]. They also stated that the synergistic effect shortens the total duration of therapy and decreases drug-related toxicities by allowing the use of lower doses. Hence, in the current system, isolating, identifying, and evaluating the promising bioactive phytoconstituents from the plant extracts become essential [43].

The present study showed that the combination of chloroform fractions of methanolic extract of *W. somnifera* and *C. roseus* with the antibiotics was more synergistic than being indifferent or antagonistic. The antibacterial combinations resulted in synergy that strongly inhibited the growth of the bacterial isolates. In accordance with our findings, Gaur et al. [44] have observed the synergistic effect of *C. roseus* against *Xanthomonas*, *P. aeruginosa*, and *S. Typhi*. In addition to this, Muddukrishnaiah and Singh [45] have reported the synergistic effect of *W. somnifera* against MDR strains of *E. coli* and *S. aureus*. The FTIR spectroscopy of the chloroform fractions of *C. roseus* and *W. somnifera* revealed the major independent peaks of probable functional groups of bioactive compounds present in the leaves of the plants.

In the present study, the major FTIR peaks were obtained at 3357 cm^{-1}, 2933 cm^{-1}, 2929 cm^{-1}, 2877 cm^{-1}, 1734 cm^{-1}, 1693 cm^{-1}, etc. which belong to the predominant functional groups, alcohols, carboxylic acids, alkanes, aldehydes, and alkyls. In accordance with our findings, Rajeev [46] obtained the FT-IR spectrum of the *Withania somnifera* (leaves) samples between 3320.29 cm^{-1}, 1652.83 cm^{-1}, 2945.67 cm^{-1}, 2834.64 cm^{-1}, 1449.39 cm^{-1}, 1113.62 cm^{-1}, 575.61 cm^{-1}, 1417.14 cm^{-1}, 1016.45 cm^{-1}, 755.15 cm^{-1}, 546.14 cm^{-1}, 534.78 cm^{-1}, and 510.12 cm^{-1}, respectively, which indicate the presence of flavonoids in the leaf extracts.

The present study gives a clue to develop new antimicrobials based on the combination of bioactive compounds and synthetic drugs, which can be used for the treatment of MDR strains and also to boost the immunity of the host. The study also highlighted the role of secondary metabolites and bioactive compound present in the plant leaves as the most effective antimicrobial constituents. The next necessary step in the study is to isolate these bioactive compounds from the fractions of medicinal plants.

5. Conclusion

The study suggested that to overcome the problems regarding the emergence of MDR strains, the plant derivatives as alternatives in combination to synthetic drugs can help in the development of much effective therapeutics to treat the MDR strains of *S. Typhi* and other bacteria. The present study also suggests that the combined treatment with these can enhance the property of the immune system to perform better action against the external pathogens as well as in the treatment of internal pathogens. The study also suggested that synergistic drugs may be the golden standard in the inhibition of protective biofilms of resistant bacteria. Further studies are needed with these herbs to isolate, characterize, and elucidate the structure of the bioactive compounds of the herbs which are responsible for the antimicrobial activity and other therapeutic value.

Data Availability

The data (figures and tables) used to support the findings of this study are included within the article as well as in the supplementary data.

Conflicts of Interest

All authors have none to declare.

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Supplementary Materials

Table 1: antityphoid/antisalmonella assay of 25 plant extracts under study. Table 2: reactivity of different antigens O, H, and AH with the serum samples by slide agglutination test in both genders. Table 3: antibiogram profile of MDR *S. Typhi* isolates. Table 4: determination of MIC of resistant antimicrobials against *S. Typhi* isolates ($n = 17$). Table 5: yield of methanolic extracts of medicinal plants obtained from dried extracts. Table 6: synergistic assay of methanolic extracts against MDR *S. Typhi* isolates. Table 7: antisalmonella activity of the various fractions of *Withania somnifera*. Table 8: antisalmonella activity of the various fractions of *Catharanthus roseus*. Figure 1: antisalmonella effect of methanolic extract fractions (Ch = chloroform; ChB = basified chloroform; Bt = butanol; EA = ethyl acetate; Aq = aqueous) against MDR *S. Typhi* isolates. *(Supplementary Materials)*

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