Synergistic Antimicrobial Activity of *Camellia sinensis* and *Juglans regia* against Multidrug-Resistant Bacteria

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Data Availability Statement: All relevent data are within the paper and its Supporting Information files.

Abstract

Synergistic combinations of antimicrobial agents with different mechanisms of action have been introduced as more successful strategies to combat infections involving multidrug resistant (MDR) bacteria. In this study, we investigated synergistic antimicrobial activity of *Camellia sinensis* and *Juglans regia* which are commonly used plants with different antimicrobial agents. Antimicrobial susceptibility of 350 Gram-positive and Gram-negative strains belonging to 10 different bacterial species, was tested against *Camellia sinensis* and *Juglans regia* extracts. Minimum inhibitory concentrations (MICs) were determined by agar dilution and microbroth dilution assays. Plant extracts were tested for synergistic antimicrobial activity with different antimicrobial agents by checkerboard titration, Etest/agar incorporation assays, and time kill kinetics. Extract treated and untreated bacteria were subjected to transmission electron microscopy to see the effect on bacterial cell morphology. Camellia sinensis extract showed higher antibacterial activity against MDR S. Typhi, alone and in combination with nalidixic acid, than to susceptible isolates." We further explore anti-staphylococcal activity of *Juglans regia* that lead to the changes in bacterial cell morphology indicating the cell wall of Gram-positive bacteria as possible target of action. The synergistic combination of *Juglans regia* and oxacillin reverted oxacillin resistance of methicillin resistant *Staphylococcus aureus* (MRSA) strains *in vitro*. This study provides novel information about antimicrobial and synergistic activity of *Camellia sinensis* and *Juglans regia* against MDR pathogens

Introduction

The increase of infections involving multidrug-resistant (MDR) bacteria and of resistance to last resort antimicrobial agents have limited therapeutic options of bacterial infections.

According to the World Health Organization, infectious diseases are the third most significant cause of mortality around the world (http://www.who.int/mediacentre/factsheets/fs310/en/index2.html). The burden of infectious diseases is high in developing countries, as is the emergence of MDR pathogens due to poor health-care facilities, and over-the-counter availability and misuse of antimicrobial agents [1,2]. The frequency of resistance is observed equally among Gram-negative and Gram-positive organisms, although Gram-negative bacteria are prone to develop a MDR phenotype. The high incidence rate of MDR *Pseudomonas* and *Acinetobacter* infections in critically ill patients as well as the presence of MDR *Salmonella* and *Staphylococcus aureus* in normal communities are classic examples of microbiological challenges posed in these geographic locations [3,4,5,6].

In the last fifty years, only two novel classes of antimicrobial compounds such as oxazolidinone and cyclic lipo-peptide have passed clinical trials and are available for clinical use. These agents have undergone analogue development and six drugs were introduced in market in the last decade; however, they are not useful for MDR pathogens due to the rapidly evolving mechanisms of resistance among bacteria [7,8]. Most other compounds do not proceed to clinical trials due to lack of sustained activity and higher toxicity rates. It is clearly understood that progress in the field of drug discovery is far behind what is required to keep up with present day needs. The situation necessitates the development of new antimicrobial agents in rapid fashion.

Plants are historically used to treat infectious diseases. In earlier days people used to discover remedies from the local herbs. People first used plants as food and if results of ingestion were favorable, the plants were linked with some sedative and curative properties. For example, remains of the hollyhock plant, which is still an important herb in phytomedicine, are found in the ancient civilization of the Neanderthals [9]. Scientific evidence supports the hypothesis that several plants are composed of biologically active chemical entities and several drugs in modern day medicine are actually analogues of plant origin substances [10,11,12]. In this study, we aimed to screen the antibacterial activity of *Camellia sinensis* (green tea) and *Juglans regia*, which are commonly used in the Pakistani population. Aqueous and methanolic preparations of plant extracts were tested for antibacterial activity against a wide variety of pathogens alone and in combination with several antimicrobial agents. We further determined their effect on bacterial morphology.

Materials and Methods

Bacterial Strains

A total of 350 Gram-positive and Gram-negative strains were obtained from the culture repository of the Immunology and Infectious Diseases Research Laboratory, Department of Microbiology, University of Karachi. Bacterial strains were of clinical and environmental origins (Tables 1 and 2). Clinical strains were isolated from urine, stool, wound, sputum and blood samples, submitted to several pathological laboratories of Karachi for microbiological culture and sensitivity. Environmental isolates were obtained from the culture collection of Department of Microbiology, University of Karachi. Reference strains included *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhi ATCC 13311, and *Shigella flexneri* ATCC 9199. Biochemical identification and antimicrobial susceptibility was performed as described previously [13] and antimicrobial susceptibility data were interpreted according to standard breakpoints described by clinical and laboratory standard institute (CLSI) criteria for each antimicrobial agents [14]. The origin and resistance pattern of these bacterial isolates are given in S1 Table. Isolates were stored in Luria-Bertani (LB) broth

Table 1. Antimicrobial activity of *Camellia sinensis*.

Organisms	Origin	R-type	n	*Camellia sinensis* (green tea)					
				Aqueous			Methanolic		
				Zone (mm)	MIC mg/ml	MBC mg/ml	Zone (mm)	MIC mg/ml	MBC mg/ml
Staphylococcus aureus	Clinical	S	40	**17**	0.78	1.56	**20**	0.39	1.56
Staphylococcus aureus	Clinical	MRSA	99	**17**	0.19	1.56	**20**	0.39	1.56
Streptococcus pyogenes	Clinical	S	05	**17**	0.78	1.56	ND	ND	ND
Escherichia coli	Clinical	S	125	07	3.12	5.0	06	5	>5
Salmonella enterica serovar Typhi	Clinical	MDR	16	06	1.56	1.56	06	1.25	2.5
Salmonella enterica serovar Typhi	Clinical	S	22	06	3.12	3.12	06	2.5	2.5
Pseudomonas aeuginosa	Clinical	MDR	02	06	>5	>5	06	>5	>5
Acinetobacter buamannii	Clinical	MDR	01	06	>5	>5	06	>5	>5
Klebseilla pneumoniae	Clinical	MDR	01	06	3.12	5.0	06	5	>5
Citrobacter freuendii	Clinical	MDR	01	06	3.12	5.0	06	5	>5
Enterobacter cloacae	Clinical	MDR	01	06	3.12	5.0	06	5	>5
Bacillus subtilis	Env.	S	02	**17**	0.78	1.56	**20**	0.78	1.56
Streptococcus pneumoniae	Clinical	S	01	**12**	1.56	3.12	**20**	0.78	3.12
Micrococcus	Env.	S	01	**13**	0.78	1.56	**20**	0.39	1.56
Salmonella Paratyphi A	Clinical	S	08	06	1.56	3.12	06	1.25	2.5
Shigella species	Clinical	S	**15**	06	3.12	6.25	ND	ND	ND
Helicobacter pylori	Clinical	R A C	02	06	>5	>5	09	2.5	>5
Helicobacter pylori	Clinical	S	03	06	>5	>5	09	2.5	>5
Campylobacter jejuni	Clinical	S	05	06	>5	>5	06	>5	>5
S. aureus ATCC 25923,	Reference			20	0.78	1.56	20	0.39	1.56
Escherichia coli ATCC 25922,	Reference			06	3.12	5.0	06	5	>5
Salmonella Typhi ATCC 13311,	Reference			06	3.12	3.12	06	2.5	2.5
Shigella flexneri ATCC 9199	Reference	S		06	>5	>5	06	2.5	>5

S_ pan susceptible; MRSA_ Methicillin resistance *Staphyococcus aureus*; MDR_ Multidrug resistant; MIC_ minimum inhibitory concentration; MBC_ minimum bactericidal concentration; Env._ environmental; R-type_ Resistance phenotype; n_number of isolates; zone_diameter of zone of inhibition; R A C_Rifampicin Ampicillin Chloramphenicol.

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containing 50% glycerol at -80°C. Log phase bacterial cultures grown in LB broth were used for antibacterial assays.

Preparation of plant extracts

Plant material included the dried leaves of *Camellia sinensis* (green tea) and the bark of *Juglans regia* (locally referred to as dandasa). The samples were purchased from a local market and identified by Ms. Muneeba Khan, the designated plant taxonomist of the Department of Botany, University of Karachi, Pakistan. Plant material was ground to a powder in a mechanical grinder. Five percent (w/v) aqueous infusion of plant material was prepared in sterile ultrapure laboratory grade water by three successive cycles of heating at 80°C for three minutes. The solutions were filtered using 0.22μm membrane [15]. For methanolic extracts, plant material was soaked into 95% methanol in a ratio of 1: 10 (w/v) for 72 hours at 22°C with vigorous shaking. The extract was subjected to methanol evaporation under vacuumed pressure and residual material was considered as source of methanolic extract. Stock solutions were prepared in DMSO

Table 2. Antimicrobial activity of *Juglans regia*.

| Organisms | Origin | R-type | N | *Juglans regia* | | | | | |
| | | | | Aqueous Crude | | | Methanol | | |
				Zone mm	MIC mg/ml	MBC mg/ml	Zone mm	MIC mg/ml	MBC mg/ml
Staphylococcus aureus	Clinical	MRSA	99	**23**	0.31	0.6	**28**	0.31	1.06
Staphylococcus aureus	Clinical	S	40	>**20**	1.25	2.5	**18**	0.53	1.06
Bacillus subtilis	Env.	S	01	**20**	0.78	2.5	**19**	1.06	2.5
Micrococcus	Env.	S	01	**19**	0.78	1.56	**19**	0.78	1.56
Escherichia coli	Clinical	S	123	06	>5	> 5	06	1.06	2.1
Salmonella enterica serovar Typhi	Clinical	MDR, S	38	**13**	>5	> 5	06	>5	>5
Pseudomonas aeruginosa	Clinical	MDR	2	06	>5	> 5	06	>5	>5
Streptococcus pyogenes	Clinical	S	05	**15**	1.25	5	**12**	1.25	5
Streptococcus pneumonia	Clinical	S	01	06	>5	>5	**12**	1.25	5
Shigella species	Clinical	S	15	06	2.5	>5	06	>5	>5
Enterobacter cloacae	Clinical	MDR	01	06	5	>5	06	>5	>5
Pasteurella multocida.	Clinical	S	02	**15**	1.25	5	09	2.5	5
Helicobacter pylori	Clinical	R A C	02	06	>5	>5	09	>5	>5
Helicobacter pylori	Clinical	S	03	06	>5	>5	09	>5	>5
Campylobacter jejuni	Clinical	S	05	06	>5	>5	06	>5	>5

S_ pan susceptible; MRSA_ Methicillin resistance *Staphyococcus aureus*; MDR_ Multidrug resistant; MIC_ minimum inhibitory concentration; MBC_ minimum bactericidal concentration; Env._ environmental; R A C_ Rifampicin Ampicillin Chloramphenicol; R-type_ Resistance phenotype; n_number of isolates; zone_diameter of zone of inhibition

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(Merck, Darmstadt, Germany)) and final working volumes were achieved by diluting the stock into Muller Hinton (MH) broth (Oxoid, Hamshire, UK).

Antimicrobial susceptibility assays

A total of 350 bacterial strains (Tables 1 and 2) were subjected to antimicrobial susceptibility and MIC assays. In addition, reference strains such as *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhi ATCC 13311, and *Shigella flexneri* ATCC 9199 were run each time as control for susceptibility and MIC assays. Plant extracts were screened for antimicrobial activity using the agar well diffusion method. Bacterial cultures were inoculated in LB broth for 3 hours at 37°C and turbidity was adjusted in phosphate buffered saline (PBS) to 0.5 McFarland's index. Using a sterile cotton swab, a bacterial lawn was spread on 90 mm MHA plates containing 6mm wells. Twenty microliters of plant extracts were poured into each well and plates were incubated at 37°C aerobically for 18 hours. Columbia blood agar plates containing 7% lake horse blood (Oxoid, Hamshire, UK) provided a platform for *Helicobacter* and *Campylobacter* based assays. Plates were incubated in microaerophilic conditions using a Campygen kit (Oxoid, Hamshire, UK) at 37°C for 48 hours. The diameter of the zone of bacterial inhibition around each well was measured [16].

Minimum inhibitory concentrations (MICs) were determined by agar dilution and micro-broth dilution assays using MH agar and MH broth respectively [17]. The concentrations of the extracts tested ranged from 5000 to 50μg/ml. In the case of agar dilution, experiments were performed on MH agar plates containing various concentrations of extracts (1:20 v/v). Plates were inoculated with ten microliters of the test organism containing 10^5 CFU log phase bacteria per spot and a total of 20 spots were tested on one plate. In the case of the microbroth

dilution assay, sterile flat-bottom 96-well plates were loaded with 100μl of two-fold dilutions of extracts into each well. The starting bacterial inoculum was 1.5×10^5 CFU/ml. Plates without any plant extracts were served as growth control. The assay plates were incubated as described above. The highest dilution of extract that showed no visible bacterial growth per spot and no turbidity in agar dilution and microbroth dilution assay respectively was considered as MIC. To determine minimum bactericidal concentration (MBC) of extracts, 100μl of MH broth from each well of microbroth assay was sub-cultured on MH agar plates after 24 hours of initial incubation. MH plates were incubated for another 24 hours. The lowest concentration of extract that resulted in no bacterial growth was considered as MBC. Experiments were performed in quadruplicate on five different occasions.

Synergistic antimicrobial assays

Antimicrobial activity of plant extracts in the presence of different antimicrobial agents, such as oxacillin, tetracycline, nalidixic acid, ofloxacin, chloramphenicol, gentamicin and penicillin was determined. The details about the prepration of antimicrobial agents are provided in S2 Table. *Camellia sinensis* was tested against representative strains of *Salmonella enterica* serovar Typhi (n = 16) while *Juglans regia* extract was tested against *Staphylococcus aureus* (n = 21). Each combination was first tested by the checkerboard titration method using MH broth in microtiter plates as described previously [18]. Concentrations of extracts ranged from 5000 to 5 μg/ml. The antimicrobial activity of the extract and antibiotic combination was interpreted as one of the following categories: Synergy; indifferent; additive effect; or antagonism. The fractional inhibitory concentration (FIC) of each agent was calculated as the MIC of the agent in combination, divided by the MIC of the agent alone. The interpretation was made on the basis of the fractional inhibitory concentration index (ΣFIC), which is the sum of the FICs of both agents. The FICI results were interpreted as follows: < 0.5 synergy; 0.5 to 1 additive effect; 1–2 indifferent or no effect; and >2 antagonism [19]. Synergistic combinations were further checked using the Etest/agar incorporation assay [20] in which an Etest-strip of each respective antimicrobial agent was placed on MH agar plates containing FIC and 0.5 x FIC of each extract. MH plates containing no antimicrobial agent or extract served as growth control. Plates were incubated at 37°C for 16 hours. Breakpoint values for the resistance of antimicrobial agents were defined per standard criteria.

Time kill kinetic assay

Time kill kinetic assays were performed on successful synergistic combinations obtained by Etest-strip and checkerboard titration methods. Flasks containing 100 ml MH broth and the drug-plant extract combination were inoculated with a log phase culture of the test organism to a density of 1×10^5 CFU/ml. Test strains included *Salmonella enterica* serovar Typhi (n = 16) and *Staphylococcus aureus* (n = 21). Individual components of each combination, either extract or antibiotic, were added to the control flask to compare the effects of the synergistic combinations to their individual effects on the bacterial growth curve, while no drug was added to the growth control flask. Flasks were incubated for 24 hours at 37°C. Aliquots (100μl) were collected at different time intervals from each flask, serially diluted in PBS, and cultured on MHA plates to obtain colony counts [21]. Curves were constructed by plotting the $\log_{10}$ of CFU/ ml versus time. Synergy was defined as $\geq 2 \log_{10}$ decreases in CFU of organisms treated with the drug combination compared to the most active component of the alone as described previously [22].

Transmission electron microscopy

On the basis of biological activity, bacterial strains treated with sub-inhibitory concentrations (0.5 x MIC) of *Camellia sinensis* and *Juglans regia* extracts were subjected to transmission

electron microscopy to see their effects on bacterial cell morphology as previously described protocol with some modifications [23]. Test organisms (*S. aureus* n = 21) were grown in 10ml of MH broth containing sub-inhibitory concentrations (0.5 X MIC) of plant extracts, at 37°C for 18 hours. Broth was centrifuged at 5000 x g and pellets were washed twice with PBS, fixed with gluteraldegyde and OsO_4, dehydrated in graded ethanol and resuspended in 500µl PBS. Formvar coated 300-mesh copper grids were coated with 5µl sample suspension for 10 min with the help of sterile tweezers and rinsed once with sterile MilliQ water to remove excessive non-coated material. Copper grids were locally made by the mechanical engineering department of the N. E.D. University of engineering, Karachi. Negative staining was performed by immersing coated grids in 1% uranyl acetate for 20 seconds followed by destaining in sterile MilliQ water and air drying. Grids were observed directly with a JOEL CO-Japan's JEM 100 transmission electron microscope operating at 80 kV.

Results

Antimicrobial activity of *Camellia sinensis* extracts

Susceptibility studies showed that C*amellia sinensis* (green tea) extracts have higher antimicrobial activity against multidrug resistant *Salmonella* Typhi and sensitive *Salmonella* Paratyphi A than other Gram-negative isolates (Table 1). The *S.* Typhi strains (n = 16) with phenotypes resistant to ampicillin (Amp), tetracycline (T), streptomycin (S), co-trimoxazole (Sxt), nalidixic acid (Na), and ciprofloxacin (Cip) were inhibited by 1.25mg/ml of methanolic and 1.56mg/ml of aqueous extract of green tea. The respective MIC/MBC ratio was 1 and 0.5, and MICs were higher to pan-susceptible strains, however no activity (only 6 mm zone of inhibition) was observed around by agar well diffusion assay. The difference in the data obtained by these assays could be explained by the presence of high molecular weight bioactive compounds which are not readily absorbed and move through solidified agar medium. Time kill kinetics further explained the bacteriostatic effect of green tea extracts on *S.* Typhi (Fig. 1A). No significant antimicrobial activity was observed in other Gram-negative and microaeophilic organisms such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter buamannii*, *Klebseilla pneumoniae*, *Citrobacter freundii* and *shigella species*. Although the MIC of aqueous extract was 3.12 mg/ml, they were not considered active due to higher MIC values in methanolic extract and no activity by agar well diffusion assay. Green tea was found to be very active against Gram-positive organisms, including *Staphylococcus aureus*, *Microccocus*, *Streptococcus pneumonia* and *Bacillus subtilis*. The methanolic extract inhibited the growth of *S. aureus* with a 20mm zone of inhibition and an average MIC of 0.39 mg/ml and MBC 1.56 mg/ml. Although insignificant, MICs of aqueous extracts were lower for methicillin-resistant *Staphylococcus aureus* (MRSA) compared to sensitive reference and clinical strains of *S. aureus* (Table 1 and S3 Table). Dose dependent cidal effect was observed against drug-resistant and sensitive strains on 24 and 6 hours respectively (Fig. 1B and 1C).

Antimicrobial activity of *Juglans regia* extracts

The most potent inhibitory effect of *Juglans regia* extracts was observed on MRSA strains with MICs 0.31 mg/ml and a zone of inhibition of more than 20 mm around aqueous and methanolic extracts (Table 2). Interestingly, two- to four-fold increases in MIC were found when drug-sensitive clinical strains of *S. aureus* were tested, but the MIC/MBC ratio was 0.5 in both cases. In contrast with green tea, *Juglans regia* did not show activity with Gram-negative bacteria; only *E. coli* was found to be susceptible at 1.06 mg/ml of methanolic extract with no zone of inhibition. Time kill kinetics showed a dose dependent bactericidal effect on methicillin-resistant and sensitive *S. aureus* strains, indicating strong anti-staphylococcal activity of *Juglans regia* irrespective of the spectrum of resistance against other antimicrobial agents (Fig. 2). Our data
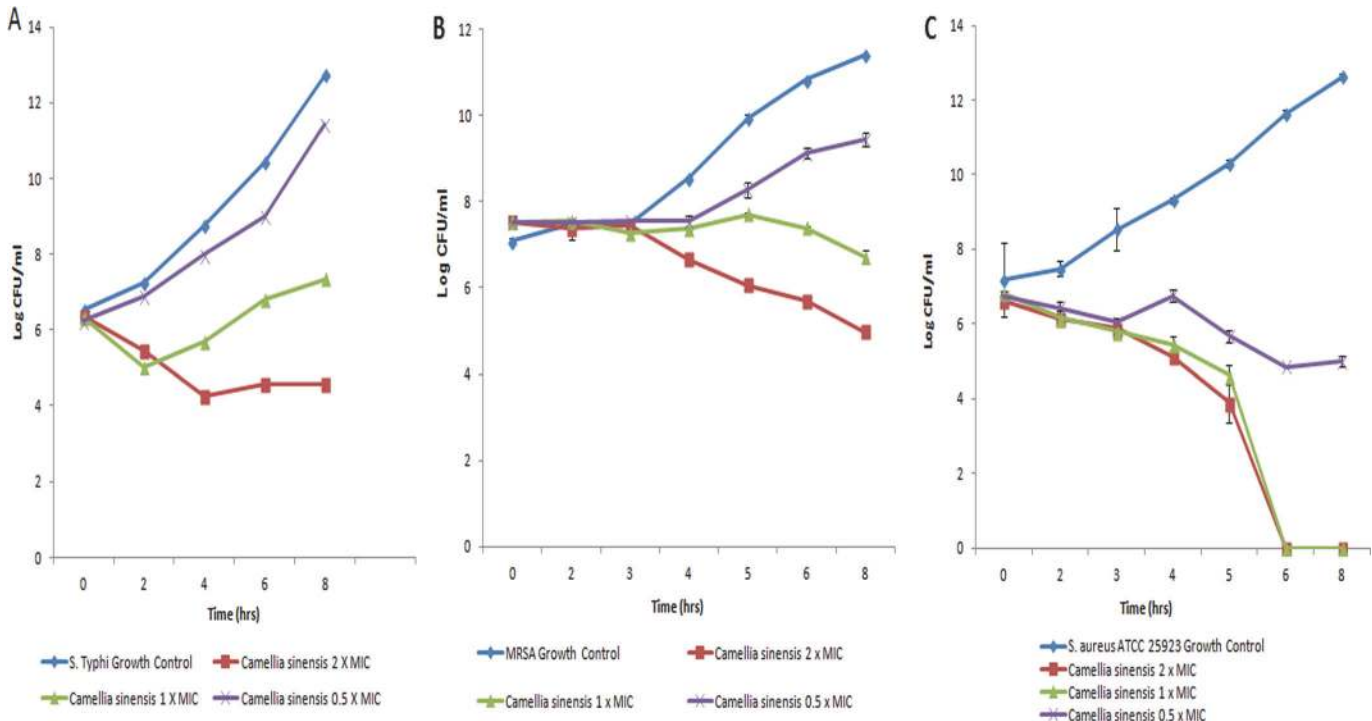
Fig 1. Effect of *Camellia sinensis* on bacterial growth kinetics. Representative bacterial strains of (a) *Salmonella enterica* serovar Typhi, (b) Methicillin resistant *Staphylococcus* aureus (MRSA), and (c) *S. aureus* ATCC 25923 (pan—susceptible) were treated with graded concentrations of methanolic extract of *Camellia sinensis* (green tea). Growth cycle of untreated organisms served as growth control. A 2 Log_{10} decrease at any time point from original CFU was considered as significant. Results are presented as an average for three experiments.

clearly indicate strong antimicrobial activity of *Juglans regia* against Gram-positive organisms, particularly *Staphylococcus* species.

Synergistic activity of plant extracts with antimicrobial agents

A number of antibiotic combinations were checked with synergistic activity of *Camellia sinensis* and *Juglans regia* extracts and two different combinations showed strong synergistic activity (Table 3). When *Camellia sinensis* was combined with nalidixic acid, it was able to inhibit *S.* Typhi at sub-MIC levels. We also observed a significant reduction in MICs of nalidixic acid, which explains the strong synergy (FICI 0.37) in this combination (Table 3). However, the MICs of nalidixic acid did not reach breakpoint level (8μg/ml) for the MDR strain (R type-Amp C Sxt T Na). Time kill kinetics showed synergy at 2, 4, 6 and 8 hours (Fig. 3A).

In the case of *Juglans regia*, 10 times reduction in MICs was observed against *Staphylococcus aureus* when used in combination with oxacillin. In this combination, oxacillin was able to inhibit MRSA strains at 0.312μg/ml, which was 64 times lower than the MIC of oxacillin alone and indicates a reversion of methicillin resistance if used along with *Juglans regia*. In time kill kinetics, the *Juglans regia* and oxacillin combination exhibited synergy at 3, 4, 5, 8 and 9 hours in 10/10 MRSA strains (Fig. 3B). A complete cidal effect was observed in 9 hours, which persisted to 24 hours of incubation.

Effect on bacterial cell morphology

Transmission electron microscopy showed characteristic morphological changes in MRSA after receiving treatment with *Juglans regia* extract for 18 hours. In contrast with untreated
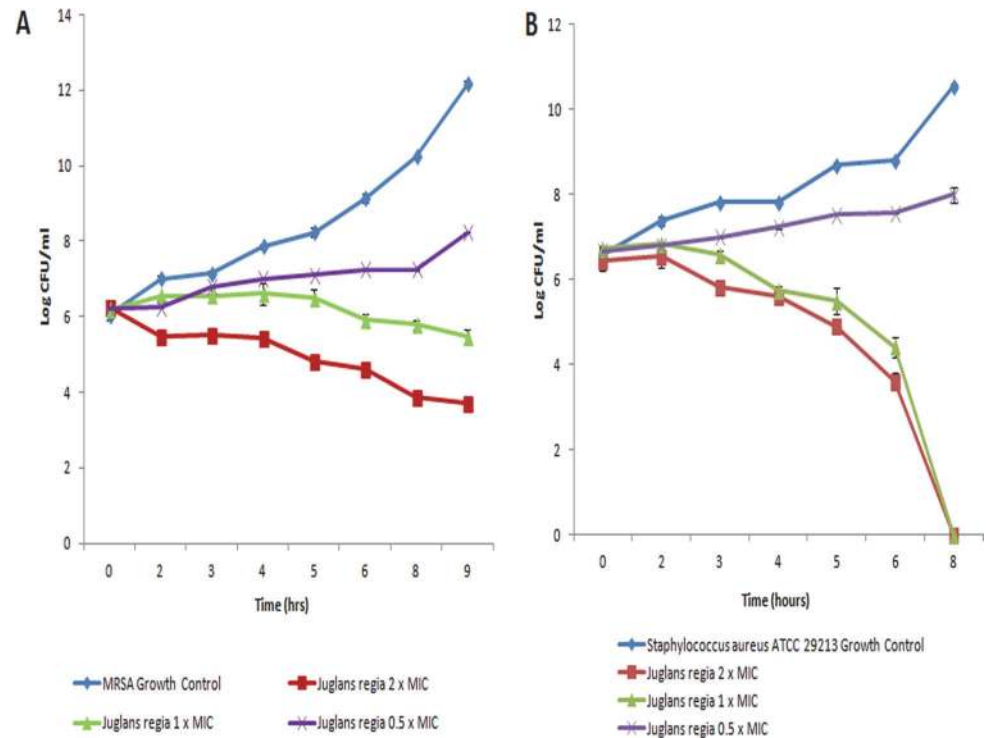
Fig 2. Effect of _Juglans regia_ on bacterial growth kinetics. (a) Methicillin resistamt _Staphylococcus aureus_ (MRSA) and (b) _S. aureus_ ATCC 25923 (pan—susceptible) were treated with graded concentrations of aqueous extract of _Juglans regia_ (Dandasa). Growth cycle of untreated organisms served as growth control. A $2Log_{10}$ decrease at any time point from original CFU was considered as significant. Results are presented as an average for three experiments.

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growth control (Fig. 4A), thick intracellular masses were observed in bacterial cells treated with _Juglans regia_ (Fig. 4B) that further led to the deformation of bacterial cells (Fig. 4C and 4D); however, we did not find complete deformation of the cell. We also observed that _Juglans regia_-treated deformed bacteria were coated with thread-like material, indicating the presence of extract on the cellular surface (Fig. 4). Prominent changes in bacterial cell morphology indicate that bacterial cell membrane permeability and viscosity was compromised by the _Juglans regia_ extract.

Discussion

Camellia sinensis belongs to the family Theaceae of tea plants. It is widely cultivated in tropical and subtropical regions all over the world. Previous studies reported the antimicrobial activity of green tea leaves to Gram-positive organisms [24,25,26]; however, discrepancies were found regarding its activity in relation to Gram-negative rods [27,28,29]. In this study, we observed that _Camellia sinensis_ showed higher antibacterial activity against MDR _S._ Typhi than to other Gram-negative isolates. By testing a variety of clinical strains in a time kill kinetic assay, we further showed that green tea extract is bacteriostatic in nature and more efficient against MDR strains of _S._ Typhi compared to pan-susceptible strains. Previous studies have reported that tea catechins are bioactive components present in green tea particularly they are active against Gram positive bacteria[30,31], however none of them is seen to be antimicrobial against Gram negative bacteria. To the best of our knowledge this is the first report providing strong evidence of the antimicrobial activity of green tea against drug-resistant Gram-negative bacteria.

Table 3. Synergistic antimicrobial activity of plant extracts with different antimicrobial agents.

Bacteria	R-type	n	Antimicrobial Agent	MIC of Antimicrobial Agent (µg/ ml)		MIC of *Camellia sinensis* (mg/ml)		FICI	Outcome
				Alone	combination	alone	combination		
Salmonella enterica serovar Typhi	AmpCSxtTNa	3	Chloramphenicol	512	256	2.5	2.5	1.5	Indifferent
			Tetracyclin	1	0.5	2.5	2.5	1.5	Indifferent
			Nalidixic acid	256	32	2.5	0.62	0.37	Synergistic
	Amp C Sxt T	7	Nalidixic acid	8	1	2.5	0.62	0.37	Synergistic
	Pan-susceptible	6	Nalidixic acid	0.5	0.12	2.5	1.25	0.74	Additive
			Tetracyclin	0.25	0.25	2.5	1.25	1.5	Indifferent
			Chloramphenicol	2	2	2.5	0.75	1.3	Indifferent
						MIC of *Juglans regia* (mg/ml)			
Staphylococcus aureus	MRSA	15	Gentamicin	32	16	0.31	0.31	1.5	Indifferent
			Penicillin	4	1	0.31	0.15	0.7	Additive
			Ofloxacin	16	16	0.31	0.31	2	Indifferent
			Oxacillin	≥20	0.312	0.31	0.039	0.14	Synergistic
			Tetracycline	64	64	0.31	0.96	4	Antagonism
	Pan-susceptible	6	Gentamicin	2	2	1.25	1.25	2	Indifferent
			Penicillin	0.05	0.02	1.25	0.65	0.92	Additive
			Ofloxacin	0.25	0.25	1.25	1.25	2	Indifferent
			Oxacillin	0.5	0.312	1.25	0.37	0.92	Additive
			Tetracycline	1	1	1.25	2.5	3	Antagonism

FICI_ fractional inhibitory concentration index; R-type_ Resistance phenotype; n_number of isolates; zone_diameter of zone of inhibition; MIC_ minimum inhibitory concentration

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Juglans regia (walnut tree) is a common temperate forest tree found in Asia. The dried bark of this treeis traditionally used in Pakistan to improve oral hygiene and promote teeth brightening. The tree is rich in polyphenols and in previous studies the antimicrobial activity of this plant was reported against bacteria and fungi [32,33,34]; however, these studies were focused on the tree's leaves instead of the bark. In this study, we tested *Juglans regia* bark extracts against a wide range of bacterial pathogens and susceptibility data showed that the extract is selectively active against Gram-positive bacteria. We observed strong anti-staphylococcal activity of *Juglans regia* that led to the changes in bacterial cell morphology. The study suggests that *Juglans regia* might affect how metabolic processes occur in bacterial cell walls and cell membranes. In contrast with the findings of Nariman et al. [35], no activity was observed against microaerophilic organisms such as *Helicobacter* and *Campylobacter*. The observations warrant further investigation for the search of compound(s) present in *Juglans regia* bark that leads to the development of novel antimicrobial agent.

In this study, we investigated antimicrobial activity of plant extracts by several methods including agar well diffusion, agar dilution and microbroth dilution assays. Significant differences were not observed in the MICs of *Camellia sinensis* and *Juglans regia* extracts obtained by agar dilution and microbroth dilution methods. However the growth of certain strains was not inhibited by agar well diffusion assay. Given that the assay was performed with crude extract, we hypothesize that presence of high molecular weight components might hinder in the uniform distribution and movement of bioactive compound through solidified agar medium.
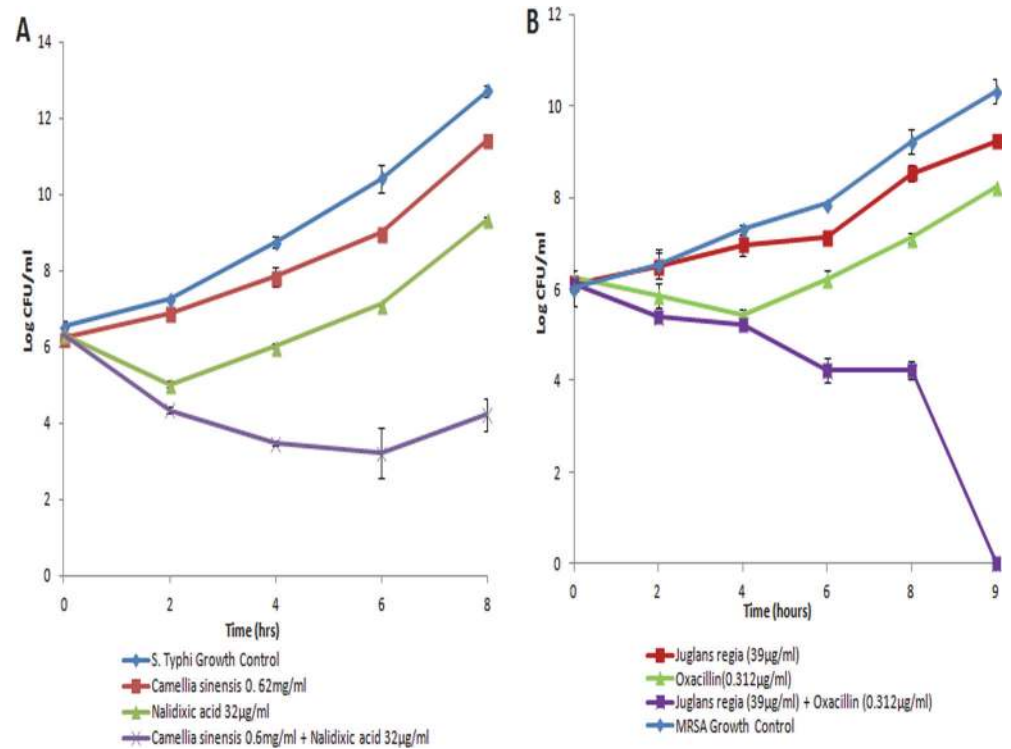
Fig 3. Effect of synergistic antimicrobial combinations on growth kinetics of MDR pathogens.
Synergistic antimicrobial combinations of (a) *Camellia sinensis* and nalidixic acid and (b) *Juglans regia* and
oxacillin were tested for antimicrobial effect on growth kinetics of MDR *Salmonella enterica* serovar Typhi and
Methicillin Resistant *Staphylococcus aureus* respectively. Organisms were grown in MH broth in the presence
of plants extracts alone and in combination with antibiotics and samples were taken for colony counts at
different time intervals. Control organisms were grown in MH broth only. A 2$\log_{10}$ decrease at any time point
from original CFU was considered as significant. Results are given as average for three experiments.

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Although the antimicrobial activity of these plants has been previously reported, little is
known about their interaction with antimicrobial agents which are commonly prescribed to
treat MDR bacterial infections. Here we tested the synergistic activity of *Camellia sinensis* and
Juglans regia extracts with a wide range of antimicrobial agents. *Camellia sinensis* showed syn-
ergistic activity with nalidixic acid (FICI 0.37) against MDR *S*. Typhi strains. Since *Camellia
sinensis* is bacteriostatic for Gram-negative rods, we hypothesize that this combination might
prevent the recurrence of bacterial growth caused by antibiotic selective mutants towards the
end of the disease's course; therefore, it is worthwhile to test *Camellia sinensis* and its com-
pounds for synergistic activity with quinolones *in vivo*.

It was very surprising to see that *Juglans regia* was able to reverse oxacillin resistance in
MRSA. Oxacillin is a β-lactam antibiotic that inhibits cell wall peptidoglycan through binding
and competitive inhibition of penicillin binding proteins (PBPs) [36]. Activation of *mecA* gene
and gene variants led to the formation of PBP2a, which binds with β-lactam antibiotics in lower
affinity, resulting in drug resistance[37,38]. Therefore, unavailability of PBP2a might resume an-
timicrobial action of oxacillin in MRSA strains. We have already shown that *Juglans regia* alone
induces ultrastructure changes in MRSA, indicating significant changes in the molecular machin-
ery of bacterial cells. We speculate that the addition of *Juglans regia* might interfere in the synthe-
sis or transportation of PBP2a levels on the cell membrane. Previous studies also showed similar
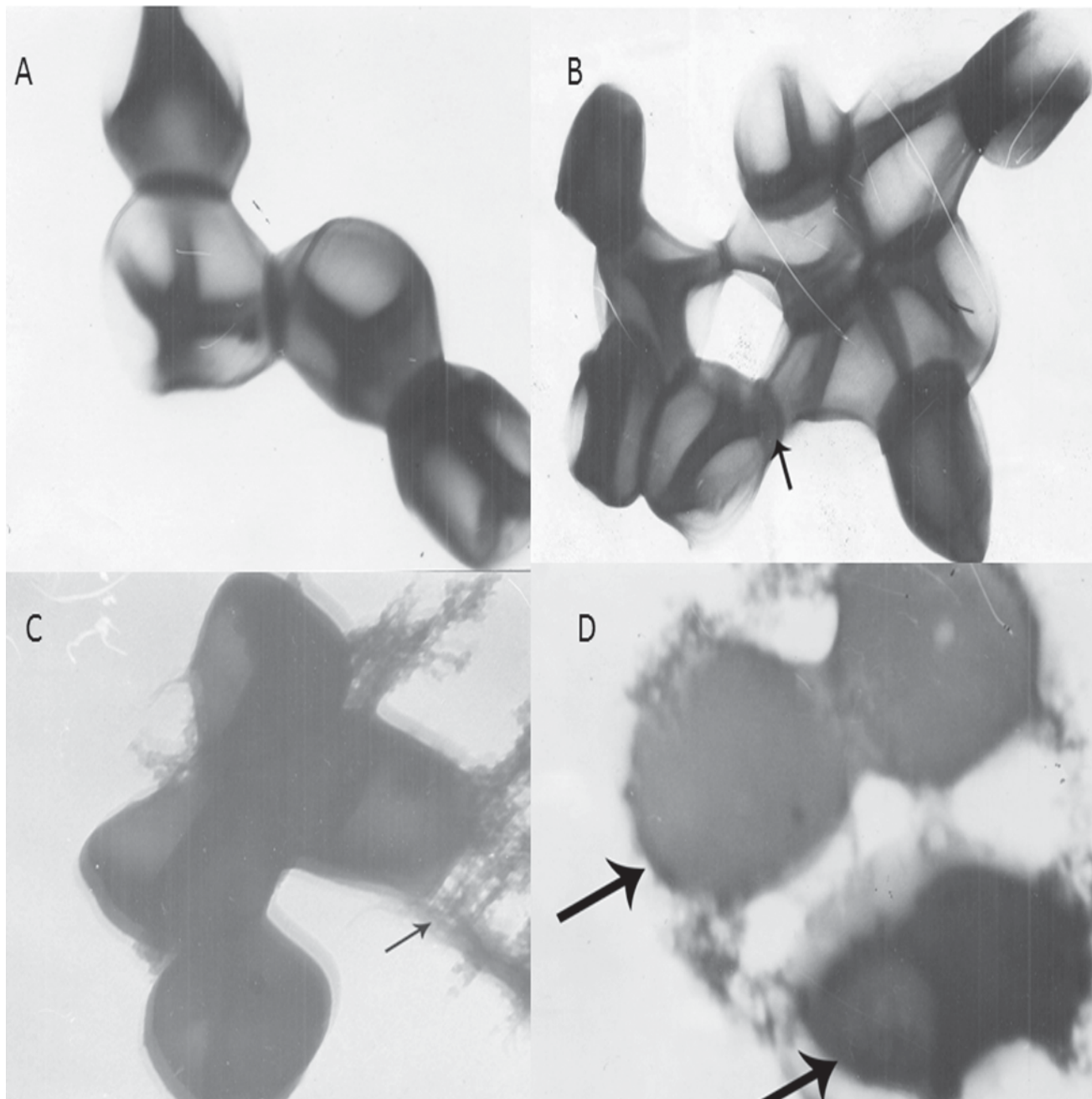effects on oxacillin resistance by different compounds such as phenothiazine, chlorpromazine,

Fig 4. Effect of *Juglans regia* on cell morphology of MRSA. Change in cellular morphology of MRSA was observed by Transmission Electron Microscopy, A: Control cell, B-D shows bacterial cells treated with *Juglans regia*. B: Intracellular thick material. C: covering on unknown material on cell surface. D: Hollow and de-shaped bacteria.

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thioridazine, inducing physiological damage to bacterial cell membranes [39,40]. Further studies are warranted to understand the exact mechanism of action. This study is the first to report the synergistic antimicrobial effects of *Juglans regia* in combination with β-lactam antibiotics leading to the reversion of oxacillin resistance.

In a separate study, we tested these extracts for toxic effects on human RBCs. *Camellia sinensis* was found to be non-toxic on the concentrations tested for antimicrobial activity while

Juglans regia showed toxicity at concentrations 100 times higher than the one showed antimicrobial activity (data not shown). The significant increase in toxic concentrations for human RBCs demonstrates the feasibility of *Jeglans regia* to test for further bioactive compound purification activities.

This study provides novel information about the antimicrobial potential of *Camellia sinensis* and *Juglans regia* to MDR pathogens. Further studies investigating the mechanism of synergistic action are important to prove candidacy of these substances for antimicrobial therapy.

Supporting Information

S1 Table. Antimicrobial resistance pattern of bacterial isolates used in this study.
(XLS)

S2 Table. Detailed information about antimicrobial agents.
(XLS)

S3 Table. Raw data for antimicrobial activities of Camellia sinensis and Juglans regia against 350 bacterial isolates.
(XLS)

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Author Contributions

Conceived and designed the experiments: AF BP SUK SR. Performed the experiments: AF BP IB AK. Analyzed the data: AF AK SR BP. Contributed reagents/materials/analysis tools: SUK SR. Wrote the paper: AF AK.

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