Research Article

Anti *Campylobacter* Activity of Extracts of *Daphne Mucronata* and *Symplocos Racemosa* Against Avian Isolates

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Abstract: Bacterial gastroenteritis mainly caused by *Campylobacter* sp. is a major health concern in Pakistan. In order to investigate the primary sources of *Campylobacter* infection, total 81 cloacal swab samples were collected from different avian species in Karachi. *Campylobacter* strains were isolated, identified and antibiotic susceptibility pattern was studied. All isolates of *Campylobacter* were sensitive to Gentamicin and Ofloxacin. It was observed that 23% of the *C. jejuni* positive isolates were resilient to tetracycline, 45% to ampicillin, and 12% to erythromycin. The *Campylobacter* isolates showed susceptibility to the extracts of two plants namely *Daphne Mucronata* and Symplocos racemosa. The sensitivity of the isolates towards these extracts can be an alternative to the traditional antibiotics for the treatment of *Campylobacter* infections.

Keywords: *Campylobacter*, avian isolates, *Daphne Mucronata*, *Symplocos racemosa*.

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INTRODUCTION

Campylobacters are micro-aerophilic, curved, non-spore forming, gram-negative rods with a corkscrew motility. They are usually present in the intestinal tract of domestic and wild animals. Birds due to their body temperature serve as favorable host and provide suitable environment for *Campylobacter* [1-3]. An estimated 20 - 40 % of sporadic disease might result from eating chicken [4, 5]. The prevalence rates of *Campylobacter* sp. from broiler flocks are 38.1- 79.2% in various European countries [6, 7].

Campylobacters are the leading source of food-borne bacterial gastroenteritis in humans [8] and also a major public health concern in Pakistan. *Campylobacter* has been reported as the third most common pathogen in stool specimens [9]. They can create a momentary asymptomatic carrier state, as well as infection (diarrhea, enteritis, extra-intestinal infections and septicemia) in humans all around the world [2]. The common species involved in *Campylobacter* infections are *C. coli, C. jejuni and C. lari.*

Although most infections by *Campylobacter* sp. do not need antimicrobial treatment but it can be fatal in erythromycin are used generally to treat *Campylobacter* infections but the resistant *Campylobacter* strains are immunocompromised patients [10]. Fluoroquinolones and emerging with an alarming rate due to excessive use of antibiotic in the feed of chicken [11] resulting in an increased risk of death [12, 13].

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With the emergence of resistant strains there is a dire need to find alternative to present drugs with high potency and reduced side effects [14]. Medicinal plants due to their excessive bioavailability provide a great alternative to find new lead molecule as an alternate and source of therapeutic agents for a very long time [15]. The plants chosen for this study, *Daphne Mucronata* and *Symplocos recemosa*, have no reported anti-*Campylobacter* activity although their antibacterial and antifungal properties towards few gram-positive and gram-negative organisms have been studied [16, 17].

This study was designed to isolate and characterize *Campylobacter* from the local poultry market of different areas from Karachi. The antibiotic resistance profile of *Campylobacter* strains was determined. Furthermore, the antimicrobial potential of the Butanolic extract, DM organic extract, Hexane fraction and water fraction of *Symplocos recemosa* and *Daphne Mucronata* against *Campylobacter* isolates was also determined. The existence of drug resistant *Campylobacter* poultry strains might draw some information on the spread of antibiotic resistance in human infections.

MATERIALS AND METHODS

Sampling

Cloacal swabs were collected from poultry retail shops as well as from the local avian market in Karachi, Pakistan. A total of 81 cloacal swabs were collected by using sterile cotton swabs. The swabs were transported to laboratory and processed for culture and *Campylobacter* isolation.

Culturing and Isolation

The cloacal swab samples were enriched for 3-4 hours in thioglycollate enrichment broth (Oxoid) containing *Campylobacter* selective supplement (Oxoid) and then 100µl of the thioglycollate broth was inoculated on modified *Campylobacter* agar base medium with addition of 5% sheep blood and *Campylobacter* selective supplement (Oxoid) to get suspected *Campylobacter* colonies. The agar plates were incubated at 42^{o}C for 48-72 hours in a microaerophilic environment (10 % CO_2, 5% O_2 and 85% N_2) by using airtight jar (2.5 liters) containing CampyGen sachets (Oxoid). Suspected *Campylobacter* colonies were further sub-cultured on Muller Hinton Agar (Oxoid) with addition of 5% sheep blood for characterization [18].

Biochemical Identification

The isolates showing characteristic *Campylobacter* growth on media plates and microscopic features were subjected to standard biochemical tests comprised of catalase, coagulase, oxidase, nitrate reduction, hippurate, H_2S production in TSI, growth at various incubation temperatures and in 3.5 % NaCl [19].

Antibiotic Susceptibility Test

Antimicrobial sensitivity pattern of the isolated *Campylobacter* isolates against antibiotics mentioned in Table **3**, were determined by the Kirby-Bauer Disc diffusion method [20]. Inoculum was prepared from a 48 hour. grown culture. An inoculum containing 10^8 cells were prepared in sterilized saline by matching the turbidity with 0.5 McFarland turbidity standard. A uniform lawn was prepared using a sterile cotton swab. Discs of respective antibiotics were placed and plates were incubated for 24 hours. under microaerophilic conditions. A zone of inhibition with diameter $\leq$10mm was considered as resistant, zone size between 10-13 mm as intermediate and $\geq$13mm was considered breakpoint for susceptibility [21, 22].

Extract Preparation

The aqueous extracts (10%) of *Daphne murconata* and Symplocos *racemosa* were prepared in distilled water by boiling the dried and chopped leaves for ~5min. The aqueous extracts were sterilized by passing through 0.22 µm membrane filters (Nalgen). Aliquots of 10 ml were prepared and stored at - 20^{o}C until use [23, 24].

The organic extracts were prepared by percolating the dried and chopped plant material at room temperature by macerating it in methanol. The methanol extracts were then dried out, dissolved in water and extracted with the desired solvents (hexane, butanol) [23, 24].

Minimum Inhibitory Concentration (MIC) of Extracts

The effect of the extracts of two medicinal plants, *Daphne murconata* and *Symplocos racemosa* was determined against the *Campylobacter* isolates. The MIC's of the extracts were determined by micro dilution method by using 96 well plate. Inoculum containing 10^8 cells was prepared from 48 hours grown cells by matching with 0.5 McFarland standard. Two-fold serial dilutions of the plant extracts were prepared in Muller Hinton broth in the range of 7.81-250 µg ml^{-1} concentration. An inoculum of *Campylobacter* isolates containing 10^6 CFU ml^{-1} cells were inoculated in each well. The controls used were Muller Hinton broth (negative control), test organism plus Muller Hinton broth (positive control) and the respective extract plus Muller Hinton broth (extract control). Minimum concentration of each extract that yielded no bacterial growth was considered as the MIC of that extract.

Statistical Analysis

All experiments were carried out in triplicate. The experimental means and standard deviations were calculated using MS Excel 2013.

RESULTS AND DISCUSSIONS

Campylobacter species are one of the prominent causes of gastrointestinal infections globally [28]. Over the last decade, the incidence of *Campylobacter* associated food poisoning has gradually increased and it is now reported to be the leading cause of bacterial gastroenteritis in the developing countries. Several studies have shown that poultry, in particular chicken, is the main source of *Campylobacter* and its meat is principally related to *Campylobacter* infection in humans [25, 26].

Present study demonstrated the significance of chickens, parrots and sparrows as reservoirs of *Campylobacter* species. On the basis of biochemical tests, *Campylobacter* species were characterized into two groups *i.e. C. jejuni* positive or negative, since *C. jejuni* is the most common gastroenteritis caused by *Campylobacter* specie. Characterization of the *Campylobacter* species is routinely done on the basis of incubation temperature of 42^{o}C to inhibit the growth of other non-thermophilic *Campylobacter* species and hippurate test [20]. Additionally, some *Campylobacter* species, e.g., *C. rectus, C. curvus, C. concisus, C. showae* and *C. gracilis* need to be incubated in a hydrogen-supplemented microaerophilic atmosphere for isolation [27].

The results indicate that out of 81 cloacal avian samples, 51 (62%) were positive for *C. jejuni* strains. Among them 30 (60%) were isolated from the cloacal swabs of chickens, 16 (32%) from parrots and 5 (10%) from sparrows (Table **1** and **2**). This indicates the high prevalence of *C. jejuni* in different avian species as demonstrated by earlier studies [28, 29].

Table 1. Phenotypic identification tests for the determination of *Campylobacter* species.

***Campylobacter* Isolates**	**Total Number of Samples**	**Biochemical Tests**								
		Catalase	**Coagulase**	**H_2S in TSI**	**Growth (3.5% NaCl)**	**Nitrate Reduction**	**Hippurate Test**	**Growth at (^oC)**		
								25	**37**	**42**
C.jejuni (positive)	51	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve
C.jejuni (negative)	30	+ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve

Table 2. Distribution of *Campylobacter* isolates among avian species.

Avian Species	**Total no of Samples**	***C. jejuni* (Positive)**	***C. jejuni* (Negative)**
Chickens	45	30	15
Parrots	27	16	11
Sparrows	09	05	04

Table 3. Antibiotics used against *Campylobacter* isolates.

Antibiotics	**Concentration (µg/ml)**
Ampicillin	25
Cephalothin	30
Erythromycin	15
Gentamycin	10
Ofloxacin	5
Tetracycline	30
Nalidixic acid	30

Epidemiology of *Campylobacter*-associated infections appear to differ between developed and developing countries. In the developed world, both chicken and adults are at risk of *Campylobacter* infection. Transmission of the bacterium has been connected to ingestion of unpasteurized milk, undercooked meat, contaminated water and travel to *Campylobacter* endemic areas [1, 3]. In contrast, in the developing world it is endemic and the infection is generally found in children, signifying that protective immunity is developed in early life due to its high level exposure [30-32].

Antibiotics not only play a pivotal role in the treatment and prevention of human and veterinary infections, they are also used in animal feed as growth promoters [33]. The increased use of antibiotics has resulted in augmented occurrence of enteric bacterial infections along with increased antibiotic resistance [34]. According to the antibiotic resistance profile observed in this study, *C. jejuni* were found to be resistant against commonly used antibiotics; Erythromycin (12%), Cephalothin (100%), Tetracylcline (23%) and Ampicillin (45%) as shown in (Fig. **1**). On the other hand, *C. jejuni* isolates were sensitive to Gentamycin, Ofloxacin and Nalidixic acid (Fig. **1**).

Although *Campylobacter*s are naturally susceptible to Fluroquinolines (ofloxacin, nalidixic acid), the resistance to these

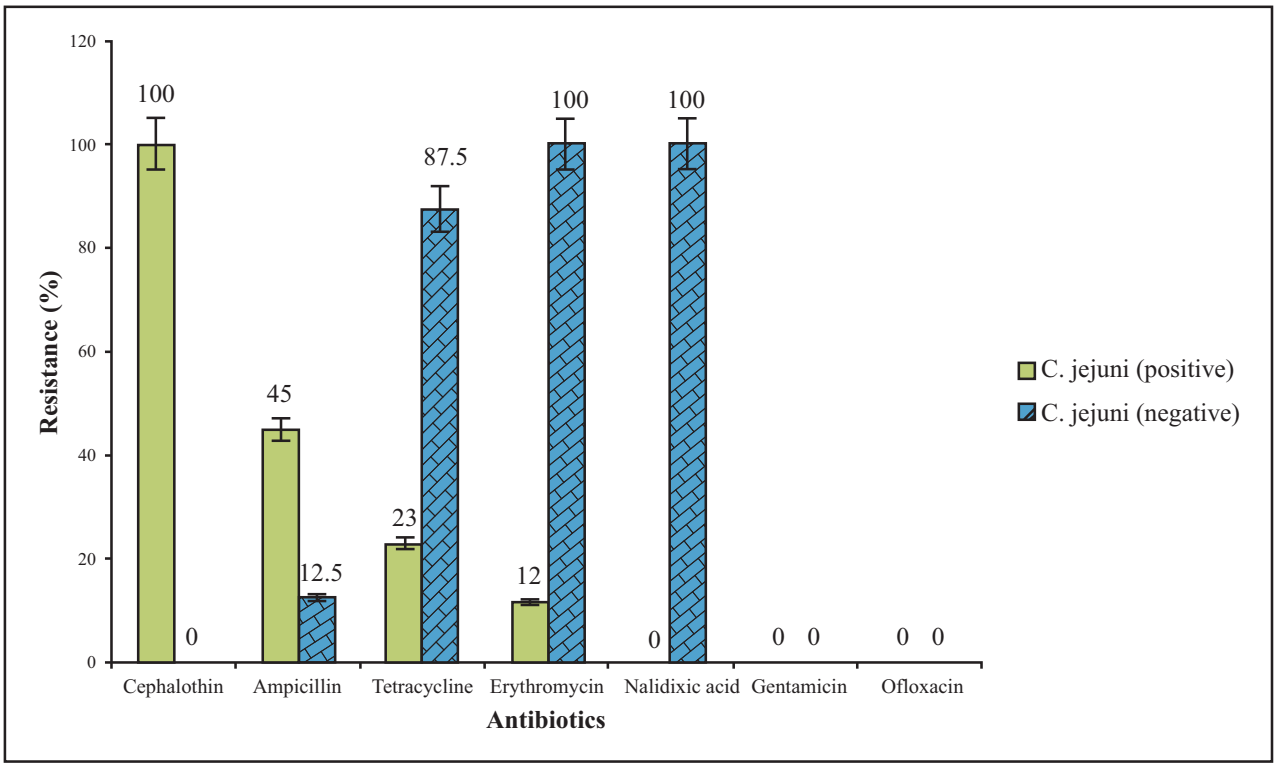

Fig (1). Antibiotic resistance profile of the *Campylobacter* isolates.

antimicrobials has amplified during the 1990s [9, 35, 36]. Increasing antimicrobial resistance has complicated the empirical treatment of Camypylobacter infections in countries where fluoroquinolone-resistant strains predominate.

A number of drugs are still effective against the flouroquinolone-resistant *Campylobacter* strains such as erythromycin (a macrolide), but higher macrolide resistance rates have been reported in some countries. According to a survey, the rate of erythromycin resistance among *C. jejuni* was 17 % in both Spain and Taiwan [37, 38]. Nalidixic acid resistant mutants of *C. jejuni* and *C. coli* showed cross resistance in a study [39]. Due to high prevalence of ampicillin-resistant *Campylobacter* species, ampicillin is not a drug of choice for *Campylobacter*iosis treatment. Tetracycline and gentamicin are recommended as alternative treatments, but they are also widely used both therapeutically and sub therapeutically as feed additive for livestock and poultry [40].

To handle antimicrobial resistant pathogen there is a dire need to find new alternative chemical entity with antibacterial action and less toxicity toward body cells. Plants provide great alternative to find new lead molecules in terms of their secondary metabolites and bioavailability. Medicinal plants were used as therapeutic agents and as a result a large number of drugs have been discovered on the basis of traditional use of medicinal plants [15, 41]. The plant-based system of infection control and treatment is still continued in the developing countries. According to a WHO report, approximately 80% of the world population relies on traditional medication for their primary health care [42]. Several studies showed the anti-microbial potentials of fruits, vegetables and plant oils which can be an alternate source for the development of new, better and non-toxic drugs [43, 44]. Moreover, different fractions extracted from plants can safely be used in tropical formulations for treating the cutaneous infections as there are no reports of harmful effects found. The natural extracts used in this study for the determination of MICs extracted from *Daphne Mucronata* and *Symplocos recemosa* have been commonly used for treating all kinds of inflammation by the local people in Northern areas of Pakistan and India [45, 46].

This work was focused on the Butanolic extract, DM organic extract, Hexane fraction and water fraction of *Symplocos recemosa* and *Daphne Mucronata*. The MICs were found to be in the range of 15.625-125 µg/ml. Butanolic extract was found to be more potent among all extracts against the *Campylobacter* isolates (Table **4**). The results strengthened the evidence of antibacterial potential of these plants [18, 17]. These variations in the antibacterial activity of the plant extracts in different solvents is dependent on the different chemical nature of the solvents [26, 36].

The results strongly suggest that these components are potential candidates for the development of new, better and

safer alternative anti-bacterial drugs that can effectively be used for the treatment of patients suffering from diseases caused by potential human pathogens like *C. jejuni.*

Table 4: Comparison of susceptibilities of *Campylobacter* isolates to the extracts of *Daphne murconata* and *Symplocos racemosa.*

***Campylobacter* Isolates**	**MIC (µg/ml)**			
	Water Extract	**Hexane Extract**	**n-Butanol Extract**	**DM organic Extract**
C. jejuni (positive)	31.25 ± 0.11	31.25 ± 0.09	15.625 ± 0.10	62.5 ± 0.12
C. jejuni (negative)	15.625 ± 0.15	125 ± 0.06	15.625 ± 0.09	125 ± 0.11

CONCLUSION

Bioactivity of the plant extracts not only provides a scientific basis for its use in traditional health care system but also suggests that these extracts or their bioactive components, if found non-toxic in animal studies and clinical trials, can serve as an effective alternative source for developing new antibiotics for the treatment of *Campylobacter* infections.

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CONFLICT OF INTEREST

Declared None.

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