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Effect of *Psidium guajava* (guava) L. Leaf Decoction on Antibiotic-resistant Clinical Diarrhoeagenic Isolates of *Shigella* spp.

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Abstract

Background: Although shigellosis is self-limiting, antibiotics are recommended to minimize the severity of symptoms and reduce mortality rates. However, due to the increasing reports of antibiotic resistance, alternative approaches are needed to combat shigellosis. Interest for research on medicinal plants has increased in recent years, and hence, they can be explored to treat this infectious diarrhoea.

Objective: To study the effect of *Psidium guajava* L. (guava) leaf decoction (GLD) on the antibiotic-resistant clinical isolates of *Shigella* spp.

Materials and Methods: A total of 43 isolated *Shigella* spp. from diarrhoeal patients were used in this study. The effect of GLD on the bacterial viability was initially assessed. The isolates were divided into two categories: sensitive and resistant to GLD. For sensitive isolates, antibacterial activity of GLD was evaluated while for resistant strains, the ability of GLD for reducing the bacterial invasion of the HEp-2 cell line underwent an investigation.

Results: Among the 43 *Shigella* isolates, GLD affected the growth of 23 strains. The invasion of 9 strains from the 20 remaining resistant isolates was unaffected. Although the number of isolates was less, the data suggested that isolates belonging to *S. flexneri* serogroup were more sensitive to GLD in comparison with other spp (i.e., *sonnei, boydii,* and *dysenteriae*).

Conclusion: The results of this study revealed the efficacy of GLD against drug-resistant *Shigella* spp. and thus could be considered for the treatment of diarrhoea. GLD can be a cost-effective alternative to antibiotics.

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Background

Antibiotics are the primary treatment for infections. However, some studies have documented the rampant and irrational use of antibiotics leading to antibiotic resistant microbes.[1-3] Considering the growing rates of treatment failures in infectious diseases, the emergence of drug-resistant pathogens is a significant threat to public health prompting the World Health Organization (WHO) to declare antibiotic resistance as a subject of global crisis.[4,5] Shigellosis, a common gastrointestinal infection, is responsible for about 700 000 deaths per year across the globe.[6] Although the infection is a self-limiting diarrhoea, it is among the few diarrhoeal diseases wherein antibiotics are recommended by the WHO for reducing the severity of symptoms and minimizing the death rate.[7] A study by Das et al associated the use of antibiotics for shigellosis to aid faster recovery and the reduction of complications and mortality.[8] With resistance developing to earlier recommended antibiotics including sulphonamides, chloramphenicol, tetracycline, and fluoroquinolones, third-generation cephalosporins were introduced for treating shigellosis.[9] However, reports of drug-resistant *Shigella* have emerged from various regions. For example, the resistance of *Shigella* to quinolones (nalidixic acid and ciprofloxacin) has been documented in the regions Europe-America and Asia-Africa.[10] In another prospective study conducted between 2009-2012 in New Delhi (India), involving 6339 stool samples from gastroenteritis patients, 121 strains (19%) were found to be *Shigella* spp. Further, 76% of these strains were multidrug-resistant, including three drugs of ceftriaxone, cefotaxime, and ceftazidime belonging to the third generation of cephalosporins.[11] In another study spanning between 2007-12, 67 isolates belonged to *Shigella* spp. These isolates showed high levels of resistance toward trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, and ampicillin. Multi-drug resistance was observed in 55%.[12] Moreover, these Indian studies,

in Shiraz, Iran, resistance of *Shigella* to co-trimoxazole was reported as 90.24%, and high resistance of *Shigella* isolates to cotrimoxazole, tetracycline, and ampicillin was shown in an Ethiopian investigation.[13,14] Therefore, multi-drug resistant *Shigella* including fluoroquinolone-resistant *Shigella* has been included in the WHO priority list (Priority 3 – medium) for research and development of new antibiotics for the treatment of infections caused by them.[2]

Psidium guajava (guava) leaves are a traditional remedy for gastrointestinal infections and have been widely reported to have antimicrobial activity.[15-17] Our previous studies with guava leaves demonstrated that its decoction acts against different diarrhoeal pathogens including *Shigella* and exhibits a wide spectrum of anti-diarrhoeal activity.[18,19] In the present study, a decoction prepared from guava leaves was tested *in vitro* for its ability to combat antibiotic-resistant clinical isolates of *Shigella* spp. Towards this, the decoction was studied for its bactericidal action and effect on the bacterial invasion of epithelial cells.

Materials and Methods

Bacterial Strains

A total of 43 clinical isolates of *Shigella* spp. were included in the study. Forty of these isolates were provided by the St. John's Research Institute (SJRI), Bengaluru, and three strains were collected from Hinduja Hospital, Mumbai. The provided strains were those received by the hospitals for diagnostic purposes and were either archival or freshly collected samples. The antibiotic susceptibility profile of the isolates was also provided by the hospitals. All the isolates were frozen in the brain heart infusion broth with 20% glycerol and stored at -80°C.

Antibiotic Susceptibility Testing

This procedure was conducted using the Kirby-Bauer method of disc diffusion as per the Clinical Laboratory Standards Institute (CLSI) guidelines.[20] Briefly, Mueller-Hinton agar (MHA) Himedia Laboratories, India was inoculated as a lawn using a pure culture of the *Shigella* isolate in peptone water after the inoculum size was adjusted to the 0.5 McFarland standard. Commercially available antibiotic discs (HiMedia Laboratories) impregnated with the recommended concentration of the antibiotics were placed on MHA. The plates were incubated overnight at 37°C, and the diameter of the zones of inhibition around the discs was measured and recorded as "resistant" or "susceptible" based on the reference table provided by CLSI. Quality control for the antimicrobial disks was performed using reference strain *Escherichia coli* (ATCC 25922). The antibiotic discs and their concentration were ampicillin (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), cotrimoxazole (23.75 µg/1.25 µg), furazolidone (50 µg), nalidixic acid (30 µg), neomycin (30 µg), norfloxacin (30 µg), and tetracycline (30 µg).

Plant Material

Mature guava leaves belonging to the *Sardar* variety were collected from Shirwal, Satara district, Maharashtra in June 2018. The leaves were authenticated by an ethnobotanist (Dr. P. Tetali), and a voucher specimen was deposited at the Naoroji Godrej Centre for Plant Research (NGCPR, Shirwal) under herbarium number NGCPR 1099.

Leaves were sent to the National Agriculture and Food Analysis and Research Institute (NAFARI, Pune) for the analysis of the guava batch obtained for the presence of aflatoxins (B1, B2, G1, & G2), heavy metals viz., tin, copper, lead, zinc, and cadmium, and the determination of the microbial load.

Preparation of Decoction

GLD was prepared by boiling 1 g of crushed leaves in 16 mL of distilled water until the volume was reduced to 4 mL. The resultant decoction was centrifuged and filtered through a 0.22 µ membrane before use. A fresh decoction was prepared for each bioassay. A few decoctions were also air-dried, and the dry weight was recorded to know the yield of the decoction.

Bio-assays

Each isolate was initially tested for growth in MHA containing 1% GLD (4 mL of freshly prepared decoction was considered 100%). Accordingly, the isolates were divided into two categories, including those - sensitive and resistant to GLD. For sensitive isolates, the antibacterial activity of GLD was undertaken, and for resistant strains, and the ability of GLD to reduce bacterial invasion was studied.

Antibacterial Activity

The antibacterial activity of GLD was assessed using the agar dilution method.[21] Briefly, bacteria grown to logarithmic phase in Luria broth were plated on MHA alone (as control) and MHA containing different concentrations of GLD (test). Bacteria showing no growth at 1% GLD were considered as sensitive, and for these isolates, the percentage decrease in bacterial growth was undertaken at 0.5% and 0.3% GLD. The colony formation units (CFUs) were enumerated following 48 hours of incubation without and with GLD. Data were expressed as percentage viability, and CFU from the control was taken as 100%. Each assay was performed in triplicates and repeated thrice.

Effect on Bacterial Invasion

The bacterial invasion of GLD resistant to 1% GLD to HEp-2 cells was assessed by the previously described method.[22] Briefly, a 48-hour culture of HEp-2 cells grown in a 24-well tissue culture plate was infected with 10^8/

mL of bacteria without (control) and with 0.25% GLD (a pre-determined non-cytotoxic dose) and incubated for 2 hours. The extracellular bacteria were washed off and the culture was further incubated with a medium containing gentamycin (100 µg/mL) for another 2 hours. Following incubation, the medium containing gentamycin was washed off, and then the epithelial cells lysed using chilled distilled water and the released bacteria were plated on nutrient agar. The CFUs were then recorded Data were expressed as percentage viability and CFU from the control was taken as 100%.

Higher doses of the antibiotic were used in clinical isolates resistant to 100 µg/mL gentamicin. However, kanamycin (150 µg/mL) was used for isolates that were highly resistant to gentamicin. The invasion rate was considered unaffected if the percentage of invasion was ≥ 50%. Each assay was carried out in duplicate wells and repeated thrice.

Results

Presence of Alfa Toxins/Heavy Metals

The report by NAFARI showed that all the tested parameters were within permissible limits or below detectable levels (data not shown).

Dry Weight of the Decoction and Percentage Yield

The average dry weight of GLD per mL and the yield were 0.013 ± 0.004 g and 5.2%, respectively.

Bacterial Isolates

Among the 43 isolates, 31 (72.09%), 8 (18.6%), and 2 (4.65%) belonged to the flexneri serogroup (*S. flexneri*), *S. sonnei* serogroup, and *S. dysenteriae*, respectively, one isolate belonged to the *S. boydii* serogroup (2.33%) while one isolate could not be characterized. The antibiotics included antibiotics in the antibiotic susceptibility testing profile were ampicillin, quinolones (ciprofloxacin, norfloxacin, and nalidixic acid), cephalosporins (ceftriaxone and cefotaxime), sulfonamide (cotrimoxazole), nitrofuran (furazolidone), aminoglycoside (neomycin), tetracycline, and chloramphenicol. Thirty-seven of the 43 (86%) isolates were resistant to ≥ 6 antibiotics. Among all the isolates, a high degree of resistance was observed for quinolones (88%) and tetracycline (83%).

Antibacterial Activity

GLD at 1% inhibited the growth of 23 isolates including 22 *S. flexneri* isolates and only one isolate belonging to *S. sonnei*. The growth of the majority of these isolates was also inhibited at 0.5% GLD. At 0.3% GLD, the bacterial viability was in the range of 0-136% (Table 1).

Effect on Bacterial Invasion

Bacterial invasion of HEp-2 cells undertaken at 0.25% GLD was in the range of 0-322% (Table 2).

S. flexneri: Among the nine strains resistant to GLD, the invasion of six strains reduced in the presence of GLD while it was unaffected in three strains. The assay could not be performed for one of the isolates as it was highly resistant to gentamicin and kanamycin. However, based on the extracellular count following infection, it was seen although the bacterial invasion decreased, the reduction was >50%.

S. sonnei: Invasion assay was performed for eight resistant strains. In three strains, the invasion was unaffected while it reduced in the remaining four strains. Amongst the three strains for which the invasion was unaffected, gentamycin was substituted by kanamycin in one strain (150 µg/mL).

S. boydii: Invasion of the single strain of *S. boydii* was reduced.

S. dysenteriae: Among the two isolates of *S. dysenteriae*, one strain being resistant to gentamicin and kanamycin, the extracellular count was estimated, and based on the

Table 1. Antibacterial Activity of GLD Against Sensitive Clinical Isolates of *Shigella*

Isolate No.	% Bacterial Viability	
	Mean ± SD [a]	
	0.5% GLD	0.3% GLD
	Shigella flexneri	
1	78 ± 4	92 ± 7
2	0	38 ± 9
3	0	48 ± 7
4	11 ± 3	56 ± 8
5	0	20 ± 5
6	0	51 ± 8
7	0	61 ± 6
8	0	84 ± 8
9	0	59 ± 6
10	0	42 ± 7
11	0	57 ± 10
12	0	29 ± 9
13	0	59 ± 8
14	0	78 ± 6
15	0	71 ± 7
16	0	86 ± 9
17	0	64 ± 3
18	0	79 ± 6
19	0	77 ± 10
20	0	78 ± 6
21	0	136 ± 7
22	0	73 ± 8
	Shigella sonnei	
23	0	0

Note. SD: Standard deviation; GLD: Guava leaf decoction; CFUs: Colony formation units.
[a] Values are represented as the Mean ± SD of CFUs from three independent experiments each carried out in triplicates.

counts, it was observed that the invasion was unaffected similar to the other isolate.

Uncharacterized *Shigella* spp.: The bacterial invasion remained unaffected in the single uncharacterized strain of *Shigella* spp.

In summary (Table 3), GLD has the potential to arrest the growth and/or reduce the invasion of a significant proportion of multidrug-resistant (MDR) *Shigella* isolates. Among the 43 isolates, GLD could arrest the growth of 23 strains (53%). The invasion of 11 strains (26%) resistant to GLD was reduced. Overall, nine strains (21%) were resistant to GLD.

Discussion

Diarrhoeal diseases continue to be a cause of global concern. Among the *Shigella* spp, *S. flexneri* is known to be the leading and common cause of diarrhoea, especially in low- and middle-income countries.[12,23] This is also observed in our study wherein the predominant strain was *S. flexneri*. This organism is highly infectious and can survive in low acidic conditions in the stomach.[23] *S. sonnei* infections are the second leading *Shigella* spp. causing diarrhoea and more commonly found in regions where there is industrialization.[24] Further, this has been observed in Vietnam and China where rapid industrialization has taken place, leading to a contaminated water supply.[25,26]

Similar to the case with other diarrhoeal pathogens, the reports of antibiotic resistance with *Shigella* spp. are on the rise. The *Shigella* isolates in the current study showed high levels of resistance toward quinolones, which was also observed in studies conducted in Bangladesh and Assam indicating resistance to ciprofloxacin and nalidixic acid.[27,28] Increased resistance to tetracycline among the applied isolates in the present study was also reported in a study conducted in Ethiopia.[29]

Due to the increasing incidences of antibiotic resistance in *Shigella* spp., there is a need for novel and efficient curative agents. Plants have been known to cater to a number of ailments, and it is estimated that 60% of the current antimicrobials are obtained from plants.[30] Hence, plant extracts have been proposed as an alternative in this regard.[31]

Psidium guajava (guava) is an important food crop that is commonly found in tropical and subtropical countries with several medicinal properties.[32] All parts of this plant are known to possess several therapeutic properties. Particularly, the leaves have been widely attributed to have varied pharmacological properties probably due to the presence of flavonoids, especially quercetin.[33,34] Our previous studies also demonstrated the anti-diarrhoeal activity of guava leaves.[18,19,35-37] These included the *in vitro* effect of GLD on the viability of *S. flexneri* and a reduction in the invading ability of the bacterium to HEp-2 cells.[18] Based on the promising results with the reference strain used, the current study was undertaken to elucidate the effect of guava leaves against drug-resistant clinical *Shigella* isolates.

The novelty of the current study lies in the fact that it explored the antibacterial activity of GLD against antibiotic resistant clinical isolates of *Shigella* spp. from India, including *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae*. Additionally, the study focused on the effect of GLD on the invasion (an important virulent feature) of *Shigella* strains whose growth was not affected by GLD in order to understand if GLD has an alternate mechanism to combat antibiotic-resistant isolates. To the best of our knowledge, this parameter has not been reported for antibiotic-resistant strains with GLD.

The results of the present study showed that GLD can arrest the growth of a significant number of clinical isolates of *Shigella*, especially *S. flexneri*. These observations are similar to available reports on the inhibitory potential of guava against *Shigella* and other drug-resistant

Table 2. Effect of GLD on the Invasion of Resistant Clinical Isolates of *Shigella* to HEp-2 Cell Line

No.	% Bacterial Invasion (0.25% GLD)
	Mean ± SD[a]
	Shigella flexneri
1	23 ± 10
2	3 ± 2
3	0
4	14 ± 9
5	26 ± 14
6	91 ± 13[b]
7	2 ± 2
8	56 ± 9[b]
9	72 ± 7*[b]
	Shigella sonnei
10	7 ± 3
11	33 ± 5
12	16 ± 9
13	15 ± 4
14	118 ± 11[b]
15	126 ± 24[b]
16	274 ± 75[b]
	Shigella boydii
17	27 ± 13
	Shigella dysenteriae
18	157 ± 56[b]
19	76 ± 7*[b]
	Uncharacterized
20	322 ± 74[b]

Note. SD: Standard deviation; GLD: Guava leaf decoction; CFUs: Colony formation units.
[a] Values are indicated as the Mean ± SD of CFUs from three independent experiments each carried out in triplicates. [b] Values ≥ 50 (invasion unaffected). *Based on extracellular counts.

Table 3. Details of Bacterial Strains and the Results of Antibacterial Activity and Inhibition of Invasion by GLD

Shigella spp	Total	Antibacterial Activity		Invasion to HEp-2 cells (0.25%)	
		Sensitive to GLD (1%)	Resistant to GLD (1%)	Reduced	Unaffected
S. flexneri	31	22	09	06	02 +1*
S. sonnei	08	01	07	04	03
S. boydii	01	0	01	01	0
S. dysenteriae	02	0	02	0	01 + 01*
Uncharacterized	01	0	01	0	01
Total	43	23/43 (53%)	20/43 (47%)	11/43 (26%)	09/43 (21%)
S. flexneri	31/43 (72%)	22/31 (71%)	09/31 (29%)	06/31 (19%)	03/31 (10%)
Others	12/43 (28%)	01/12 (8%)	11/12 (92%)	05/12 (42%)	06/12 (50%)

*Based on extracellular counts.

pathogens such as methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*.[38-41] Interestingly, the lack of the inhibition of GLD against *S. dysenteriae* isolates is also reported in a study performed in India.[42]

Several studies have indicated that the enhancement of virulence and increased antibiotic resistance could occur simultaneously.[43-45] The fundamental event in the pathogenesis of *Shigella* is its ability to invade, and subsequently, colonize the human intestinal epithelium.[46,47] The presence of several virulence factors and toxins are associated with the pathogenicity of *Shigella* spp.[48,49] Bloody diarrhoea, which is a critical condition observed in shigellosis, is induced by the *sen* gene and has been reported across all species of *Shigella*.[49-51] It has been reported that dehydration in shigellosis leading to watery diarrhoea is potentially caused by the *set1B* gene predominantly found only in *S. flexneri*.[52,53] Recent studies have shown that *S. flexneri* possesses more virulent factors compared to other *Shigella* spp.[53,54] Several studies also indicated that *Sat* and *ShET1* toxins which may be major contributors to the virulence of *S. flexneri* strains were predominantly found in *S. flexneri* but absent in other *Shigella* spp.[50,51,55,56] Although it cannot be stated conclusively, based on the above-mentioned discussion and the results of this study, it is likely that GLD selectively targets virulence factors predominantly found in *S. flexneri* leading to a decrease in their virulence.

The findings of the current study also revealed that GLD reduced the invasion capabilities of eight (90%) out of nine tested *S. flexneri* strains, followed by *S. sonnei* wherein four (58%) out of seven tested strains represented a reduction in the invasion. A plausible reason might be that GLD targets the Type III secretion system (T3SS) used by *Shigella* spp. to invade human mucosal cells.[57] This hypothesis is supported by Honma et al[58] and Michel-Briand et al[59] reporting that erythromycin selectively reduced the invasion rate of *S. flexneri* and *S. sonnei* by passing through the T3SS, as well as a recent study which suggested that T3SS in MDR *S. flexneri* harbours more virulence factors in comparison to their sensitive counterparts.[60] Thus, virulence factors in MDR *S. flexneri* targeted by GLD would result in a reduction in the invasion rate.

Therefore, it is proposed that multiple components of GLD probably act at multiple targets including virulence genes such as *set1B*, *Sat*, and *ShET1* harbored in antibiotic-resistant *S. flexneri,* along with the Type III secretion system, eventually affecting the bacterial survival and the ability to invade epithelial cells. Subsequently, it would also be worth exploring the differential expression of genes associated with bacterial growth pathways and/or bacterial virulence in the presence of GLD using transcriptomics. This would not only contribute to understanding the molecular basis of the anti-diarrhoeal activity of GLD against *Shigella* species but also provide enlarged insights into the mechanisms underlying its efficacy against drug-resistant clinical isolates of this bacterial species.

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Authors' Contributions

P. D. and V. M. undertook the bioassays, carried out the literature survey, and drafted the manuscript. R. M. and M. D. provided the clinical *Shigella* isolates and were responsible for the antibiotic susceptibility testing of the isolates. T. B. was responsible for the study, contributed to the study design, and was involved in the preparation of the manuscript.

Ethical Approval

This study was approved by the Ethics Committee of Foundation for Medical Research (Reference: FMR/IREC/MP/01/2019) and the St. John's Research Institute (Reference: IEC/1/354/2019).

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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