ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 12, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

BERBERINE HYDROCHLORIDE COULD PROVE TO BE A PROMISING BULLET AGAINST *CLOSTRIDIUM DIFFICILE* INFECTION: A PRELIMINARY STUDY FROM SOUTH INDIA

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Received: 02 August 2017, Revised and Accepted: 25 September 2017

ABSTRACT

Objective: Recurrent *Clostridium difficile* infection (CDI) and the emergence of strains with reduced susceptibility to metronidazole and vancomycin warrants alternative therapy. Hence, we tested the potential efficacy of the natural compound berberine hydrochloride (BBRHCl) against toxigenic *C. difficile*.

Methods: Three representative polymerase chain reaction confirmed, toxin-positive strains were included in the study. Pulsed-field gel electrophoresis (PFGE) profile and antibiogram of the strains were analyzed along with 10 other toxin positive isolates. Efficacy of BBRHCl against toxigenic *C. difficile* was determined using agar diffusion by punch well method.

Results: PFGE grouped the test strains into three clusters with unique susceptibility pattern toward standard antibiotics. BBRHCl was efficacious against the test strains at a concentration ranging between 6.25 µg/ml and 10 mg/ml. BBRHCl's breakpoint point inhibitory zone diameter was equivalent ($p<0.001$) to the epidemiological cutoff values for teicoplanin, vancomycin and 2% black seed oil. Although the predicted concentration of BBRHCl for breakpoint zone diameter equivalent to European Committee on Antimicrobial Susceptibility Testing's epidemiological cutoff value for metronidazole was observed to fall outside the tested concentration range; it was still within the safe dosage for humans.

Conclusion: The present study is promising in considering BBRHCl as a potent substitute or adjunct not only for metronidazole, vancomycin and teicoplanin but also for natural compounds like 2% black seed oil for managing resistant cases of CDI. Owing to BBRHCl's direct antibacterial and anti-inflammatory action, further investigations will aid in the proper characterization of the therapeutic effects of similar plant compounds, to develop safe and effective drugs against the epidemiological outbreak of CDI.

Keywords: Berberine hydrochloride, *Clostridium difficile*, *Clostridium difficile* infection, Diarrhea, Minimum inhibitory concentrations, Pulsed-field gel electrophoresis.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i12.21732

INTRODUCTION

Clostridium difficile associated diarrhea (CDAD) is one of the most dreaded infectious conditions in hospitalized patients. The infection by this Gram-positive spore bearer occurs after long-term administration of antibiotics when the normal gut microbiota gets depleted paving the way for this opportunistic pathogen.

To a large extent, vancomycin and metronidazole work well as the drugs of choice for treating CDAD with exceptions of a few cases of recurrence as observed with vancomycin [1]. Due to this problem of recurrence, researchers have been on the lookout for alternative treatment modalities for curing *C. difficile* infection (CDI).

Since the increasing interest in complementary medicine, berberine, an isoquinolone alkaloid has attracted attention as a potential alternative medical therapy [2]. It can be found in the roots, rhizomes and stem barks of several plants such as *Berberis vulgaris* (barberry), *Berberis aquifolium* (Oregon grape), and *Hydrastis canadensis* (goldenseal). Berberine can also be isolated from seeds of *Argemone mexicana*, which grows as weeds on roadsides and cultivation fields in the temperate regions of India [3]. This alkaloid has been used for several decades in herbal medicine to treat intestinal parasitic infections, diarrhea caused by bacteria, ocular trachoma infections to name a few. One of the many mechanisms that are attributed to its efficacy includes decreasing the enterotoxin-mediated secretion of water and electrolytes [2,4].

Berberine hydrochloride (BBRHCl) ($C_{20}H_{17}NO_4HCl$), one of the derivatives of berberine, has also been extensively used in China for many years as a gastrointestinal remedy and is known to possess antibacterial, antifungal, and anti-inflammatory as well as anticancer activity [5-7]. This prompted us to carry out a preliminary study to observe the effect of BBRHCl on the clinical strains of toxigenic *C. difficile* isolated from hospitalized patients with diarrhea from this region of South India.

METHODS

Bacterial strains

Three representative bacterial strains used in this study were the clinical strains of *C. difficile* isolated from polymerase chain reaction confirmed toxin-positive stool samples of hospitalized patients with

diarrhea admitted to a tertiary care hospital in Udupi district of Karnataka, South India. Pulsed-field gel electrophoresis (PFGE) was performed with these strains along with 10 other toxin-positive strains at the National Institute of Cholera and Enteric Diseases Kolkata, following pulse net protocol as described in our previous study [8,9]. Briefly, plugs were prepared using 1% SeaKem gold agarose (Sigma, St Louis, MI). The restriction enzyme used was at a concentration of 50 U *SmaI* (New England Biolabs, Ipswich, MA) in 0.6 ml of restriction enzyme buffer (×1.0). PFGE was performed using a CHEF-Mapper (BioRad, Hercules, CA) at 6V/cm for 18 hrs at 14°C at an angle of 120°C (initial switch time, 6.76 seconds; final switch time, 38.35 seconds). The electrophoresis buffer had 200 µM thiourea added to avoid the degradation of DNA. Gels were stained with ethidium bromide and visualized under UV light. Gel images were normalized against peaks of Salmonella enterica serotype Braenderup H9812 size standard and analyzed using BioNumerics software version 5.0 (Applied Maths, Austin, TX). A dendrogram showing the hierarchical representation of the level of linkage among the isolates was drawn to demonstrate their degree of clonal relatedness.

Inoculum preparation

A loop full of isolated colonies from a pure growth of *C. difficile* on sheep blood agar was inoculated into 4 ml of Brucella broth and incubated at 37°C (preferably with shaking) for 24-36 hrs.

The bacterial suspension's turbidity was matched to the standard 1 McFarland unit (1 ml of 1.175% [w/v] barium chloride dihydrate in 99 ml 1% [v/v] sulfuric acid). This turbidity was equivalent to approximately 3×10^8 organisms/ml [10,11]. This suspension was used for further testing on specific susceptibility testing medium.

Media used

Brucella agar plates supplemented with 5% Laked Horse Blood (Oxoid, UK), 1% Vitamin k and Hemin (BD Difco) was used for susceptibility testing using commercially available BBRHCl (Sigma-Aldrich (USA). Initially, a stock solution of 100 µg/ml was prepared by dissolving the purified BBRHCl in dimethyl sulfoxide (DMSO-Sigma-Aldrich).

Doubling dilutions were tested starting from 50µg/ml to 1.56 µg/ml.

Later a stock solution of 10 mg/ml was prepared following the procedure as mentioned earlier and three ranges of concentrations were tested as follows:

- 100-275 µg/ml (100,125,150, 175, 200, 225, 250, and 275)
- 125-1000 µg/ml (125, 250, 500, and 1000) and
- 1.25 mg/ml to 10 mg/ml (1.25, 2.5, 5, and 10).

Determination of antibacterial activity

Antibacterial activity was tested by employing the "punch-well" technique involving agar diffusion method. The test inocula were then swabbed uniformly onto Brucella blood agar plates supplemented with 1% Vitamin K and Hemin. Wells of diameter 6 mm were punched out in each plate using sterile cork borer (6 mm in diameter). 70 µl of each of the dilutions were pipetted into these wells, and the plates were incubated upright at 37°C for 48-72 hrs using Gas-Pak (BD Difco) in an anaerobic jar. The control plate had DMSO alone added into the well. The sensitivity of different bacterial strains was calculated by measuring the diameter (in millimeters) of the zone of inhibition. Readings were taken at the end of 48hrs. *C. difficile* showing a clear zone of inhibition >8 mm was considered to be sensitive. In our study, the zone diameter breakpoint of >8 mm (diameter of inhibition zone) was considered significant based on recently published literature on disk diffusion susceptibility testing for *C. difficile* using natural extracts as well as European Committee on Antimicrobial Susceptibility Testing (EUCAST's) recommendation for determining the epidemiological cutoff values and disk diffusion correlates [12,13]. Experiments were performed in triplicates for each combination of the concentrations with BBRHCl. The inhibitory zone diameters (corresponding to the test concentrations of BBRHCl) were compared with EUCAST's epidemiological breakpoint diameters for metronidazole (23 mm), vancomycin (19 mm), and susceptibility breakpoint diameter of teicoplanin (15 mm), from published literature against *C. difficile* [14,13]. The disk potency of all the three drugs (metronidazole, vancomycin, and teicoplanin) was 5 µg as reported in literature [14,13].

The potencies of BBRHCl for each of the test concentrations were calculated as a function of the volumes of drug used at a given concentration for agar diffusion method.

However, only those test concentrations have been represented whose zone diameters had a significant difference in comparison to the published literature on the effect of 2% black seed oil against *C. difficile*. Hence, the different potencies of BBRHCl represented were 87.5 µg for 1.25 mg/ml, 175 µg for 2.5 mg/ml, 350 µg for 5 mg/ml, and 700 µg for 10 mg/ml (Fig. 1).

Sensitivity of bacteria to standard antibiotics

The minimum inhibitory concentrations (MIC) of the six commonly employed antibiotics, namely, metronidazole, vancomycin, ampicillin-sulbactam, clindamycin, moxifloxacin, and imipenem were performed (following clinical and laboratory standards institute guidelines) by the E-test method for clinical isolates of *C. difficile* [11]. Further, we estimated the concentration of BBRHCl that could be used to generate a diameter of the zone of inhibition to comply with the EUCAST's epidemiological cutoff value for metronidazole (23 mm) for toxigenic *C. difficile*.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., Version 7, La Jolla, CA, USA) with all data represented as the mean ± standard deviation from at least three independent experiments. R^2 was computed as a measure of goodness of fit in a linear regression model to estimate the fraction of the total variance of BBRHCl's inhibitory zone diameter that can be explained by the change in its concentration. Extrapolation algorithm of GraphPad Prism was used to estimate data from linear regression line fitting data points, representing BBRHCl's observed inhibitory zone diameter plotted against its concentration range. Data analyses were performed using one-way analysis of variance (ANOVA) and $P\leq0.05$ was considered statistically significant.

Similarity analysis of PFGE gel images was performed with Dice's coefficient, and clustering was performed by unweighted pair group mean association [15,16].

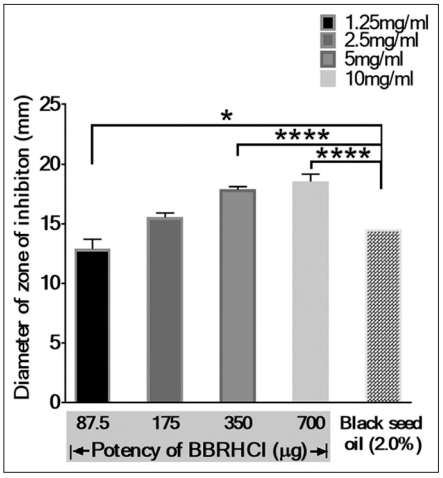

Fig. 1: Comparison of the inhibitory zone diameters (mm) for varying concentrations of berberine hydrochloride with the susceptibility breakpoint zone diameter of 2% black seed oil (15 mm from literature) for toxigenic *Clostridium difficile*. (*P≤0.05, ****P≤0.0001; n=3)

Ethical considerations

The study protocol was reviewed and approved by the Institutional Ethics Committee (Kasturba Hospital, Manipal University, Manipal (IEC 87/2012)).

RESULTS

The three representative toxin-positive isolates (designated as RC2, RC3, and RC6) were grouped into three different clusters A, B, and C, respectively, on dendrogram analysis of PFGE along with the 10 other toxin-positive strains. RC 2 belonged to Cluster A which had two strains in total with >65% similarity, RC 3 belonged to a subset of Cluster B having three strains with >85% similarity, and RC 6 belonged to Cluster C which again consisted of two strains totally with 50% similarity. These three representative strains differed in their antibiograms and their MIC along with the mean zone diameters when treated with BBRHCl have been represented (Table 1).

All tested strains were susceptible to metronidazole (MIC ≤8 µg/ml), vancomycin (MIC ≤2 µg/ml), ampicillin (MIC ≤2 µg/ml), and ampicillin-sulbactam (MIC ≤8 µg/ml).

Moxifloxacin resistance (MIC >32 µg/ml each) was detected in all the three strains. Clindamycin and imipenem resistance (MIC >256 µg/ml and >32 µg/ml, respectively) was observed in two of the three strains and tetracycline resistance (MIC >4 µg/ml) in one of the three strains. The clinical isolates of *C. difficile* responded to BBRHCl in a variable manner (Fig. 2).

The solvent control, DMSO (100%) used in our study, showed no antimicrobial effect without any zone of inhibition against the test organism.

The curve is fitting the data points in Fig. 2 demonstrated a non-linear regression between the inhibitory zone diameters and the log concentrations of BBRHCl (mg/ml) used in our study. A steep increase (slope increased from 1.045 to 4.286 (mm/[mg/ml]) in the inhibitory zone size was observed in the concentration ranging from 50 µg/ml to 1 mg/ml in all the isolated clinical strains tested. Significant difference was observed between the inhibitory zone diameters for 1.25 mg/ml (12.87 mm) when compared with 2.5 mg/ml (15.57 mm) (P≤0.01), 5 mg/ml (17.88 mm), and 10 mg/ml (18.52 mm), respectively, (P≤0.001) (Fig. 2). Similarly, there was significant difference in diameters of zones of inhibition for 2.5 mg/ml (15.57 mm) when compared with 5 mg/ml (17.88 mm) (P≤0.05) and 10 mg/ml (18.52 mm) (P≤0.01), respectively (Fig. 2). However, the difference in zone diameters was not statistically significant between the concentrations 5 mg/ml and 10 mg/ml.

The diameters of zones of inhibition observed with all the test concentrations of BBRHCl used in our study were compared with the recently published data using 2% black seed oil (susceptibility breakpoint zone diameter = 15 mm) against toxigenic *C. difficile* [12]. We observed that the diameters of zones of inhibition for concentrations 5 mg/ml (potency 350 µg) and 10 mg/ml (potency 700 µg) were found to be significantly larger (P<0.0001) than that reported for 2% black seed oil (Fig. 1).

However, the diameters of zones of inhibition corresponding to all concentrations of BBRHCl below 1.25 mg/ml (potency 87.5 µg) were found to be significantly lesser (P≤0.05) compared to that reported for 2% black seed oil (Fig. 1). The inhibitory zone diameters of BBRHCl used, in our study, were compared with that of teicoplanin (mean inhibitory zone diameter 15 mm) [14] and the breakpoint correlates for the EUCAST epidemiological cutoff values of metronidazole and vancomycin (mean inhibitory zone diameters being 23, 19 [mm], respectively) [13]. The diameters of zone of inhibition for BBRHCl (all the test concentrations) were significantly lesser (P≤0.0001) than the EUCAST epidemiological cutoff value for metronidazole (data not shown). However, EUCAST's epidemiological cutoff value for vancomycin was found to be significantly larger (P≤0.0001) than

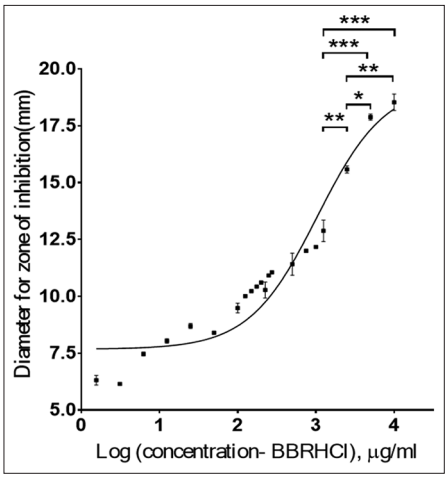

Fig. 2: Non-linear regression line obtained by plotting the inhibitory zone diameters against the log concentrations of berberine hydrochloride (mg/ml) used in the study. (*P≤0.05, **P≤0.01, ***P≤0.001; n=3)

Table 1: PFGE profile, antibiogram and diameters (mean±SD) of zones of inhibition (various statistically significant concentrations of BBRHCl) of the three representative *C. difficile* toxin-positive strains

Cluster differentiation and susceptibility pattern of the isolates	Strain 1 (RC2)	Strain 2 (RC3)	Strain 3 (RC6)
PFGE cluster	A	B	C
MIC (µg/ml)			
Ampicillin (AM)	1.0	1.5	1.0
Ampicillin-sulbactam (AB)	0.25	0.25	0.19
Vancomycin (VA)	1.0	0.25	1.5
Metronidazole (MZ)	0.25	0.19	0.38
Tetracycline (TC)	0.032	4	8
Clindamycin (CM)	0.19	>256	>256
Imipenem (IP)	>32	>32	0.094
Moxifloxacin (MX)	>32	>32	>32
Resistance pattern	IP, MX	CM,IP,MX	CM,TC,MX
Diameter of zone of inhibition for different conc. of BBRHCl (mm) (mean±SD)			
1.25 mg/ml	12.88±0.818		
2.5 mg/ml	15.54±0.358		
5 mg/ml	17.88±0.231		
10 mg/ml	18.53±0.631		

PFGE: Pulsed-field gel electrophoresis, BBRHCl: Berberine hydrochloride, SD: Standard deviation, *C. difficile: Clostridium difficile*, MIC: Minimum inhibitory concentrations

the inhibitory zone diameter corresponding to all test concentrations of BBRHCl lower than 2.5 mg/ml (potency 175 µg). A significantly larger (P≤0.0001) inhibitory zone diameter was observed for the concentrations 5 mg/ml (potency 350 µg) and 10 mg/ml (potency 700 µg) of BBRHCl against that of teicoplanin. However, inhibitory zone diameters corresponding to all concentrations of BBRHCl below 1.25 mg/ml (potency 87.5 µg) were observed to be significantly lesser (P≤0.01) than that of teicoplanin (Fig. 3).

The test concentrations of BBRHCl, lower than 1.25 mg/ml were not considered since they were significantly lesser than (i) EUCAST's epidemiological cutoff value for metronidazole and vancomycin [17], (ii) susceptibility breakpoint zone diameter for teicoplanin [14], and (iii) susceptibility breakpoint zone diameter for 2% black seed oil against toxigenic *C. difficile* [12]. Hence, a trend line (linear regression) with R^2 value 0.9506 was generated for the diameter of zones of inhibition (mm) plotted against log concentrations (1.25, 2.5, 5, and 10 [mg/ml]) of BBRHCl for toxigenic *C. difficile* (n=3). The estimated log concentration for BBRHCl corresponding to the inhibitory zone diameter of 23 mm (i.e., the EUCAST's susceptibility breakpoint diameter for metronidazole against *C. difficile*) was 1.62 mg/ml (i.e., 41.68 mg/ml) (Fig. 4).

DISCUSSION

The onset of *C. difficile* infection roughly coincides with the broad-spectrum antibiotic therapy employed for treating either diarrhea or infections due to other pathogens. Judicious use of antimicrobials is, therefore, critical in a case of *C. difficile* infection. Traditional Chinese medicine (TCM) based therapies have shown to be economical with lesser side effects in comparison to modern medicine [12]. Most of the pharmaceuticals prescribed in advanced countries are compounds derived from plants or plant products [3]. Improvement in the recovery of gastrointestinal function was demonstrated in preclinical and clinical studies using TCM based therapies [17-21].

Published literature provides evidence regarding the use of BBRHCl as an antidiabetic, antibacterial, antifungal, and anti-inflammatory agent [5], but there are hardly any reports from India regarding its efficacy against *C. difficile*. To the best of our knowledge, this study provides the first data throwing light on the potential effectiveness of BBRHCl against toxigenic *C. difficile*. Interestingly, though the three test strains considered in the present study were isolated from a single tertiary care hospital, the lack of interclonal relatedness was substantiated by their unique resistance patterns. However, all the three test strains being susceptible to BBRHCl suggest its increased efficacy against *C. difficile* over standard antibiotics. We propose that owing to the antibacterial as well as anti-inflammatory properties, BBRHCl bypasses the multifactorial mechanisms of resistance (adopted by *C. difficile*) which are otherwise effective against standard antibiotics. Inhibition of Gram-positive bacterial sortase by berberine can be attributed partially to its direct antibacterial activity toward toxigenic *C. difficile* [22]. Berberine's mechanism of action is different when compared to metronidazole and vancomycin as mentioned earlier [2,4]. This fact is of particular importance when considering various drug combinations (example non-antibiotic candidate drug with an antibiotic) where literature reports say that successful combinations can be achieved if the combination partner acts on a different molecule than that of the antibiotic [22]. Moreover, due to its anti-inflammatory activity by antagonizing lipopolysaccharide signaling, decreasing neutrophil infiltration and downregulating proinflammatory genes subsequent to TLR4–MD2 binding and inhibition of TLR4–NF-κB–MIP2, respectively, berberine qualifies as a more potent alternative to metronidazole and vancomycin in CDI management [22].

The results of the present study showed that the linear portion of the graph depicted in Fig. 2, corresponding to the concentration range 0.05 mg/ml to 10 mg/ml is statistically best suited (can generate a significantly greater response to a small change in dose of BBRHCl) for the identification of effective dose range of BBRHCl. This information can be used for comparisons or synergistic combinations with antibiotics/natural compounds against *C. difficile*. Although the lower concentrations of BBRHCl (below 0.05 mg/ml) produced the requisite susceptibility breakpoint diameter considered in our study (>8 mm), we deduce that those concentrations do not qualify for dose selection for the susceptibility testing of *C. difficile*.

The statistically validated observations of this study revealed that BBRHCl's concentrations 5 and 10 (mg/ml) corresponds to EUCAST's epidemiological cut-off diameter for vancomycin and the concentrations

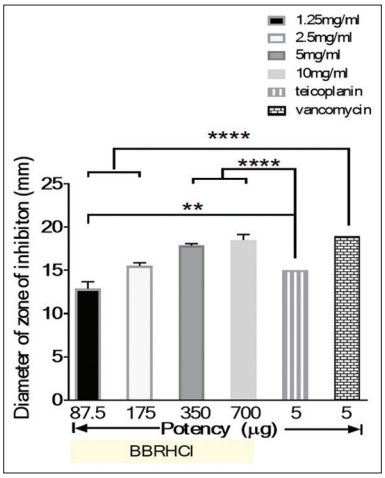

Fig. 3: Comparison of the inhibitory zone diameters of berberine hydrochloride with that of teicoplanin (mean inhibitory zone diameter 15 mm from published literature) and the breakpoint correlate for European Committee on Antimicrobial Susceptibility Testing's epidemiological cutoff value for vancomycin (mean inhibitory zone diameter being 19 mm) (P≤0.01, ****P≤0.0001; n=3)**

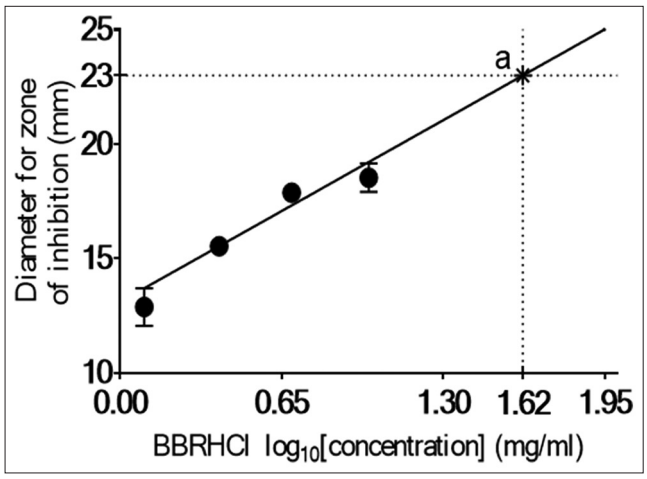

Fig. 4: Prediction of berberine hydrochloride (BBRHCl's) concentration corresponding to the European Committee on Antimicrobial Susceptibility Testing (EUCAST's) epidemiological cutoff value for metronidazole (23 mm) against toxigenic *Clostridium difficile*. The graph depicts the linear regression curve fitting the data points representing BBRHCl's inhibitory zone diameter for *C. difficile*, plotted against its corresponding log concentrations (equivalent to 1.25, 2.5, 5, and 10 mg/ml, respectively). *a: Corresponds to the concentration of BBRHCl (41.68 mg/ml) that can generate EUCAST's epidemiological cutoff value for metronidazole against toxigenic *C. difficile* which is statistically estimated by extrapolating the linear regression curve of the graph

2.5, 5, and 10 (mg/ml) to the susceptibility breakpoint diameter of teicoplanin (the standard antimicrobial agents used for treating CDI) [14]. This finding is noteworthy especially in present day scenario with emerging *C. difficile* isolates showing reduced susceptibility to metronidazole [23]. We suggest that subject to further studies, BBRHCl could be a possible substitute for vancomycin as well as teicoplanin in the case of metronidazole resistance.

Our study revealed the possibility of effective synergism between BBRHCl and 2% black seed oil against toxigenic strains of *C. difficile*. In this context, it is worth mentioning that an optimal concentration of BBRHCl (2.5 mg/ml-10 mg/ml) can prove to be beneficial (statistically validated in our study) if combined with 2% black seed oil. We suggest that the rational combination of BBRHCl and 2% black seed oil can lead to the identification of appropriate dose ratio to achieve maximum response against *C. difficile* with minimal untoward reactions to the host. Moreover, we also infer from our results that the highest test concentrations (5-10 mg/ml) of BBRHCl have the potential to substitute standard antimicrobials such as vancomycin and teicoplanin especially in cases of metronidazole resistance, for the management of CDAD.

We infer that the highest potency of BBRHCl (used in this study) found to be within the clinically recommended safe dosage of berberine for humans can be used in combination or as a substitute for vancomycin (in resistance cases) for CDI [13,24]. Although one of the limitations of this study was that the inhibitory zone diameter produced by the highest concentration of BBRHCl was significantly lower than EUCAST's epidemiological breakpoint diameter (23 mm) for metronidazole, the estimated concentration (log 1.62 mg/ml, i.e., 41.68 mg/ml as statistically predicted) of BBRHCl was still within the safe recommended human dosage [24].

The results of our preliminary study on the efficacy of BBRHCl against toxigenic *C. difficile* appear to be encouraging. It also highlights the use of agar diffusion through punch well method as a simple susceptibility testing method for this nosocomial pathogen for routine microbiology laboratories as mentioned in previous literature [13,14].

A recent report on increased uptake of berberine by germinating *C. difficile* spores suggested that at high concentrations it might inhibit the spontaneous outgrowth of the spores [25,26]. Although berberine has not been subjected to the rigorous, clinical trials; this report once again beckons the need to consider BBRHCl as a probable candidate in the management of CDI [27]. Hence, further research will enable us to formulate the most appropriate dose of berberine required to treat CDAD.

CONCLUSION

Toxigenic *C. difficile* being an emerging fastidious pathogen in the hospital settings as well as in the community requires a particular emphasis regarding therapeutics. The combination therapy of antibiotics with phytochemicals derived from plants maybe promising in the management of the multidrug-resistant enteric pathogens as evidenced from published literature [28]. Extensive studies are, therefore, warranted for proper characterization of the natural compounds to explore their therapeutic/chemical effects in the host, which in turn will enhance patient care through the production of safe and effective drugs [29]. This will help reduce the morbidity associated with CDI apart from minimizing the duration of hospital stay which poses an economic burden on the patients.

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