African Journal of
Clinical Medicine and Pharmacy Research

ISSN: 3434-2990

Index: Harvard, Boston, Sydney University, Dimensions, Lens, ResearchGet Scilit, Semantic, Google Scholar, Base etc

https://doi.org/10.58578/AJCMPR.v2i1.4720

In-vitro Cytotoxic and Anti-vibro Cholerae Activities of Methanol Roots Extracts of *Morinda citrifolia L (Noni)*

Isaac John Umaru[1], Daniel Konka[2], Iseko Iyoko Kingsly[3], Solomon Ossom Asare[4], Okrah George Hassan[5], Joseph Oteng[6], Tensaba Andes Akafa[7], Kerenhappuch Isaac Umaru[8]

[1,7,8]Federal University Wukari, Taraba State, Nigeria; [2]Ghana Health Service Municipal Health Directorate, Dormaa Ahenkro-Bono Region, Ghana; [3]Limi Hospital Abuja, Nigeria; [4]Assinmsn Nursing and Midwifery Training College, Assin Foso Ghana; [5]College of Health Yamfo, Ghana; [6]Ghana Health Service, Public Health Department, Ghana
umaruisaac@gmail.com

Article Info:

Submitted:	Revised:	Accepted:	Published:
Dec 19, 2024	Jan 3, 2025	Jan 15, 2025	Jan 20, 2025

Abstract

Knowledge on medicinal properties of plants has extensively been applied all over the World to cure or ameliorate suffering of mankind. The use of remedies from plant origin covers a wide variety of maladies and constitutes an alternative way to antibiotics, which is otherwise no longer promising due to the generalized antibiotic resistance among pathogenic microorganisms. The majority of epidemic Vibrio cholerae strains have become resistant to multiple antimicrobial agents for many reasons, including mutations, horizontal gene transfers, etc. Therefore, secondary means of treatment are needed to overcome this impediment in combating cholera. *Morinda citrifolia* roots extensively used in traditional medicine. Therefore, the present study aims to evaluate the In-vitro Cytotoxic and Anti-vibro Cholerae Activities of Methanol Roots Extracts of *Morinda citrifolia* against V. cholerae isolates. Methods: Initially, different concentration of the extracts materials was subjected to qualitative phytochemical screening. Anti-V.cholerae activities were assessed by

AJCMPR Journal is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License

performing MIC (minimum inhibitory concentration) test using disc diffusion method with different concentrations (1.25, 2.25, 5.00, 8.00, 10.00mg/ml) where standard discs of gentamycin and meropenem (10 µg/disc) were used. Further, Brine shrimp lethality bioassay was done to evaluate the extracts' cytotoxic activities using different concentrations (5,10, 20, 50, 100, 200, 400, 800µg/ml) against vincristine sulphate. Results: The preliminary screening confirms the presence of convenient phytochemical constituents like flavonoids, alkaloids, glycosides, tannins, etc., and are liable for the emergence of the antibacterial activities in different strains. The minimum inhibitory concentrations of methanolic *Morinda citrifolia* roots was found to be (a) 1.25, (b) 2.25, (c) 5, (d) 8, and (f) 10 mg/ml, respectively. Moreover, 100% mortality was observed only at the highest concentration (800 µg/ml). In comparison, the rest of the test concentration showed less than 90% mortality at the highest concentration (400 µg/ml). Conclusions: The *Morinda citrifolia* roots possess significant phytochemicals and showed potential anti-V. cholerae activities with mild toxicity. Hence, *Morinda citrifolia* (L.) roots may be recommended as potential phytotherapy against cholera disease.

Keywords: *Vibrio cholerae*, *Morinda citrifolia* (L.), roots, Minimum inhibitory concentration, Zone of inhibition, Brine shrimp, lethality assay, Cytotoxicity

INTRODUCTION

Over the years, a great deal of academic and clinical research has been done to understand, control, and contain Cholera. Cholera is recognized as an ancient diarrheal disease that is a continuous burden to public health for years in the developing and under-developed countries of Asia and Africa, where there are a population influx and poor water supply and basic hygiene. It is a water-borne bacterial disease caused by a facultative rod-shaped gram-negative anaerobic bacterium, V. cholerae. This bacterium is one of the most infamous enteric pathogens that belongs to the family Vibrionaceae [1]. The primary route of transmission of the bacterium to the human body is via the consumption of contaminated water and food. The bacterium attaches itself to the human small intestine. Eventually, it releases cholera toxins or choleragen, produced by tcp gene of V. cholerae, which results in the production of the enzymatically active subunit that elevates intracellular cAMP (cyclic adenosine monophosphate), leading to excessive loss of water and electrolytes from the body via vomiting and severe form of acute secretory diarrhea [2, 3]. However, not all V. cholerae secret cholera toxins strains.

There are only two causative strains of V. cholerae for the disease that belongs to the serogroups O1 and O139 and have been responsible for massive cholera pandemics for years [4]. The potential consequences of untreated cholera complications involve severe dehydration that may be fatal [5]. As a result, cholera is one of the leading causes of global mortality among infants, children under 5, and elderly individuals [6]. According to WHO (World Health Organization) report, every year, more than one million people suffering from acute diarrheal cases are caused by V. cholerae infection [7].

Antibiotics have been considered as the leading therapies for any kind of microbial infection. However, indiscriminate use of antibiotics may develop multi-drug resistant strains of microorganisms [11]. Nevertheless, many bacteria, including V. cholerae produce a biofilm under hostile conditions for which they show much resistance to antibiotics [12]. Besides, bacteria may develop antimicrobial drug resistance either by undergoing genetic mutation when exposed to antibiotics to treat epidemics or by the possession of resistance genes [13].

The latter is the case of V. cholerae resulting in its ability to resist certain antibiotics, including sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin [14]. Overall, the drug resistance to human pathogenic bacteria has been an alarming incidence throughout the world, raising constant demand to develop new and effective therapeutics [15]. Moreover, there is very little information about antibiotic susceptibility of environmental isolates of V. cholerae [16]. As a result, it is wise to acknowledge traditional medicine as an alternative option for treating infectious diseases as the herbal products are more economical and have fewer side-effects compared to synthetic drugs [17].

Many medicinal plants possess significant bioactive compounds that are potentially responsible for vibriocidal activities and hence recommended as promising sources of medicaments for cholera disease caused by several Vibrio spp. strains [18]. Thus, In-vitro Cytotoxic and Anti-vibro Cholerae Activities of Methanol Roots Extracts of Morinda citrifolia.

Morinda citrifolia , the plant as an edible and medicinal tropic plant -Morinda citrifolia L (Noni) The ancestor s of Polynesians are believed to have brought many plants with them, as food and medicine, when they migrated from Southeast Asia 2000 years ago[19]. Of the 12 most common medicinal plants they brought, Noni was the second most popular plant used in herbal remedies to treat various common diseases and to maintain overall good

health[20]. Noni is the common name for Morinda citrifolia L It has been reported to have a broad range of health benefits for cancer, infection, arthritis, diabetes, asthma, hypertension, and pain [21]. The Polynesians utilized the whole Noni plant in their medicinal remedies. The roots, stems, bark, leaves, flowers, and fruits of the Noni plant are all involved in various combinations in almost 40 known and recorded herbal remedies [22]. Additionally, the fruit is eaten for health and food.

Morinda citrifolia fruit has a long history of use as a food in tropical regions throughout the world. Written documentation of the consumption of this fruit as a food source precedes the twentieth century the leaves could be used as emergency food. The fruit juice is in high demand in alternative medicine for different kinds of illnesses such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digest ion, atherosclerosis, blood vessel problems, and drug addict ion. Scientific evidence of the benefits of the Noni fruit Juice is limited but there is some anecdotal evidence for successful treatment of colds and influenza[16].

However, this report intends to demonstrate the antimicrobial effect of Methanol Roots Extracts of Morinda citrifolia on Cholera. Here are the results of an in vitro antimicrobial assay from Morinda citrifolia roots extract of this plant on pathogenic strains of Vibrio cholerae, which represent the pandemics of the many years.

MATERIALS AND METHODS

Chemical reagents

Standard antibiotic discs of Gentamycin and Meropenem were procured. Müeller-Hinton agar (CMO337) was purchased from Oxoid, UK. Ethanol, methanol, tween 80, and all other reagent materials were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Brine shrimp eggs.

Microorganisms

The epidemic strains of Vibrio cholerae were used throughout this study. Table 1 shows the corresponding phenotypes

Preparation of plant extracts

Fresh Morinda citrifolia roots extract were gathered and cut to small pieces and oven-dried for 48 hrs, at 50 ºC . The material was powdered and 50 g Methanol-extracted (100 mL) at room temperature, for a week. After extraction and evaporation, the resulting solid material was collected, weighted and suspended in sterile water, up to 100 mg/mL (w/v) for further assays.

$$\% \text{ of Yield } = \frac{Weight\ of\ dried\ extract\ (gm)}{2!\,Weight\ of\ powered\ crude\ plant\ roots\ (gm)} x\ 100$$

Preliminary phytochemical screening

Medicinal plants are the potential sources of significant organic compounds like carbohydrates, alkaloids, tannins, triterpenoids, glycosides, flavonoids, steroids, and their secondary metabolites, which exert definite biological or pharmacological actions on the human body [23]. Therefore, the present investigation was continued with a qualitative phytochemical screening of freshly prepared Methanol Roots Extracts of Morinda citrifolia using standard protocols [23,24].

Bacterial strain and inoculum preparation

The Microbiology Federal Medical Centre Yola provided V. cholerae strain. The organism was immediately sub-cultured by transferring into a solid media of Müeller-Hinton agar slants under aseptic conditions followed by overnight incubation at 37 °C to confirm bacterial colonies' growth. The inoculated plants were used for examining antibacterial activities of different extracts concentration Methanol Roots Extracts of Morinda citrifolia against V. cholerae.

Screening for anti-Vibrio cholerae activities of the test extracts

Initial quantitative screening for anti-V. cholerae activities of Methanol Roots Extracts of Morinda citrifolia was performed by disc diffusion method [25]. This was done by transferring one loopful of culture from the slants to separate test tubes containing 5 ml sterile water, and the vortex eventually mixed the inoculums. About 100 µl inoculum of the bacteria was evenly spread over a media of solid Müeller-Hinton agar set on four Petri dishes with the help of a sterile cotton swab. In due course, sterilized discs of Whatman filter paper of 8mm in diameter were prepared by soaking with 40 µl of ethanolic and

methanolic extracts of Methanol Roots Extracts of Morinda citrifolia (L.) whole plant and. The final concentration of each disc was 8 mg/ml. For better precession, each concentration was tested with four discs placed on the agar medium and pressed softly by employing sterile forceps.

Standard antibiotic discs of Gentamycin and Meropenem with the strength of 10 µg/disc were represented as the positive control. The Petri dishes were then incubated for 24 h at 37 °C to let the colonies of V. cholerae evolve.

The organism's susceptibility towards various test extracts was interpreted by determining the diameter of the zone of inhibition around each disc with Vernier caliper's help [26].

Minimum inhibitory concentration (MIC) assay

The standard disc diffusion procedure was followed to determine the minimum inhibitory concentration of different test extracts against V. cholerae [27]. The bacteria colonies were collected from the subculture media and emulsified with 5 ml sterile water followed by vortex for 30 s. Subsequently, 1 ml of the inoculum was transferred and streaked using a sterile cotton swab into 150mm culture plates holding 5mm of Mueller-Hinton agar with a pH adjusted to 7.4. The plates were sterilized and individually labeled for each test extracts before the experiment.

Paper discs (8mm in diameter) of different concentrations of the test extracts ((a) 1.25, (b) 2.25, (c) 5, (d) 8, and (f) 10 mg/ml) were prepared by diluting the roots extracts of Morinda citrifolia (L.) with 0.2% Tween 80 with distilled water.

The discs were then applied and pressed gently on the culture plates using sterilized forceps. Next, the plates were subjected to incubation for 24 hrs at 37 °C. After incubation, the zones of inhibition formed around each disc were estimated on the culture plates' underside with the Vernier scale. All experimental tasks were performed under aseptic conditions.

Brine shrimp lethality assay (BSLA)

The Brine shrimp lethality test is a simple and useful benchtop method that has been considered a general bioassay for detecting wide-ranging bioactivity of natural compounds in medicinal plants and indicating the general toxicity level crude plant extracts [28].

The method is rapid, inexpensive, and easy to perform as it utilizes simple zoological organisms (Brine nauplii). For the present work, the in-vitro cytotoxicity study of different

crude plant extracts was done by applying Brine shrimp lethality bioassay based on a published protocol with a slight modification [29]. Artificial seawater was prepared by dissolving 38 g iodine-less sea salt in 1 ltr distilled water followed by filtration and filtrate collection into a large plastic container. The brine shrimp eggs were hatched in the artificial seawater with sufficient UV and oxygen supply for 2 days allowing them to thrive as nauplii. The test extracts were serial diluted with seawater to different concentrations (5,10, 20, 50, 100, 200, 400, 800µg/ml) from a stock solution of 1600 µg/ml. The stock was prepared by dissolving 32 mg of crude extract in 200 µl 0.2% Tween 80, and the final volume was made up to 20 ml with the addition of seawater. Finally, 2.5 ml of test extract from each dilution was added to 2.5 ml of seawater containing 10 nauplii in separate test tubes with a final volume of 5 ml and allowed to incubate under sufficient illumination at room temperature for 24 h. After incubation, the total number of shrimp (nauplii) survived was recorded by viewing under a magnifying glass against a dark background. The experiment was done in a triplicate.

The effect of cytotoxicity was assessed by determining LC50 (median lethal concentration or the concentration at which 50% mortality of brine shrimp nauplii occurred) values using regression analysis and percentage of mortality by applying the given formula.

$$Percentage\ mortality\ (\%) = \frac{Number\ of\ dead\ nauplii}{2!Total\ number\ of\ nuuplii}\ x\ 100$$

The data was compared against different concentrations (10, 5, 1, 0.5, 0.25, 0.125 and 0.06 µg/ml) of vincristine solution as a standard control. The experiment was validated by considering a blank (negative control) containing 4.95 ml seawater with 10 nauplii where 50 µl of 0.2% Tween 80 was added.

Data interpretation

The experimental data were represented as mean ± SEM (standard error mean) of four and three equivalent estimations for MIC (minimum inhibitory concentration) and cytotoxic assays. Regression analysis was done for assessing the values acquired from brine shrimp lethality bioassay.

RESULTS

Extraction yield of plant extracts

The extractive values (% of yield) and physical appearances of methanolic extracts of Methanol Roots Extracts of Morinda citrifolia (L.) have been represented in Table 1. The maximum percentage (8%) of the extract was obtained from the crude powder of Morinda citrifolia (L.) whole plant yielding adequate extracts for the research work. The extracts appeared to be of different colors and consistency.

Table 1: the extractive values and physical appearance of Methanol Roots Extracts of *Morinda citrifolia* (L.)

Sample	Weight of the crude extract (gm)	% yield (w/w)	Appearance
Methanol Roots Extracts of *Morinda citrifolia* (L.)	12	8	Deep brown with a citrus smell.

Table 2: Presence of Phytochemicals Methanol Roots Extracts of Morinda citrifolia (L.)

Preliminary phytochemical screening								
Test	Carbohydrate	Alkaloid	Glycoside	Tannins and Phenolic	Flavonoid	Terpenoid	Saponin	Steroids
Constituents	+	+	+	+	+	-	+	+

Evaluation of phytochemical content Preliminary phytochemical screening of the methanol roots extract of *Morinda citrifolia* (L.) revealed significant bioactive constituents, including alkaloid, glycosides, tannins, phenols, flavonoid, saponin, and carbohydrates (Table 2).

Determination of anti-Vibrio cholerae activities

Primary screening for antibacterial activities of different test concentration with 8 mg/ml potency was performed against V. cholerae. Various degrees of the zone of inhibition was observed in different samples compared with standard disks of antibiotics, including gentamycin and meropenem with a strength of 10 µg/disk (Table 3). In a recent study, the strains of V. cholerae were reported to be well susceptible to several antibiotics, including meropenem, gentamycin, ciprofloxacin, chloramphenicol, azithromycin, etc. [30]. A similar

observation was obtained in the present investigation for the antibiotics, meropenem, and gentamycin. V. cholerae was found to be susceptible to all test concentration.

Table 3: Antimicrobial screening test of methanol roots extract of Morinda citrifolia (L.) (8,6, and 4 mg/ml, 40 µl/disk) against Vibrio cholerae

Bacterial	Plant extract			Gentamicin	Meropenem
	(8mg/ml)	(6mg/ml)	(4mg/ml)		
Vibrio cholerae	13.89±1.73a(**)b(η)	11.25±0.63a(**) b(η)	12.00±0.27a(**) b(η)	22.38 ± 0.47	12.94 ± 1.66

Data represented as mean ± SEM (n = 4)

SEM standard error mean, n number of samples, at concentration of 8,6,4 mg/ml. **p < 0.01 indicates Dunnett's multiple comparison test. a = when compared with Gentamicin, b = when compared with Meropenem. (η) indicates statistically no significant difference from Meropenem

However, the highest inhibitory zone of diameter (13.89 ±1.73mm) was exhibited at 8mg/kg which corresponded to standard meropenem data.

Analysis of MIC assay

The MIC (minimum inhibitory concentration) data showing inhibitory zones of growth for different extracts are represented in Table 4. Variation in anti-V. cholerae activities of distinguished concentration has been noted in our experiment. The organism showed no sensitivity to the lowest concentration (1.25 mg/ml). Nevertheless, the minimum inhibitory concentration of 2.50 mg/ml was reported for have a clear zone of inhibition of 8.30 ± 0.36 (Fig. 1). On the other hand, a little higher concentration (5, 8, and 10mg/ml) was seen to be the maximum attentiveness for suppressing V. cholerae growth (Fig. 1), (Table 4).

Table 4: Zone of inhibition in diameter (mm) of different concentration of methanol roots extract of Morinda citrifolia (L.)

Extract	Concentrations (mg/ml) (diameter: mm)				
Morinda citrifolia (L.)	1.25	2.50	5.00	8.00	10.00
	0.00 ± 0.00	8.30 ± 0.36	12.75±1.32	13.75 ± 1.65	17.00 ± 0.41

Data represented as mean ± SEM (n = 4)

SEM standard error mean, n number of samples, mm represent as Millimeter, of methanol roots extract of Morinda citrifolia (L.) at various concentration Table 4 and Figure 1

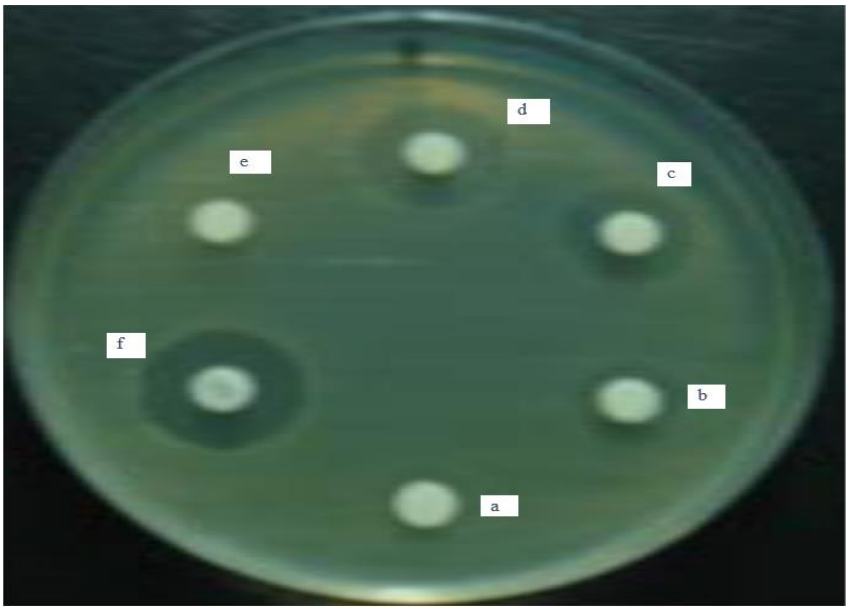


Figure1: Images of MIC (minimum inhibitory concentration) test. were (e) control, (a) 1.25, (b) 2.25, (c) 5, (d) 8, and (f) 10 mg/ml of methanol roots extract of Morinda citrifolia (L.) Each sample was replicated as n = 4. The zone of inhibition is as indicated .

Assessment of cytotoxic activity

The percentage of brine shrimp nauplii mortality was observed to be directly proportional to the concentrations of test extracts (Table 5). 100% mortality was found in the methanol roots extract of Morinda citrifolia (L.) at its highest concentration (800 µg/ml).

A similar observation was made for the case of vincristine sulphate. The lowest lethality concentration (LC50) was appeared to be made by methanolic extract of methanol roots extract of Morinda citrifolia (L.), showing a result much higher than the standard vincristine sulphate. As no mortality was seen in the negative control group, the test was valid, and the data drawn from the experiment were only due to test agents' effect.

Table 5: The percentage mortality of brine shrimp nauplii after treating with different concentrations of methanol roots extract of Morinda citrifolia (L.) (n = 3) and the standard vincristine sulphate

Conc. (µg/ml)	Log C	Morinda citrifolia	Vincristine sulphate		% mortality
			Conc. (µg/ml)	Log C	
5	0.80	10	0.06	-1.25	10
10	1.10	30	0.125	-0.90	10
20	1.40	40	0.25	-0.60	40
50	1.70	50	0.5	-0.30	60
100	2.00	60	1	0.00	70
200	2.30	60	5	1.00	100
400	2.60	60	10		100
800	2.90	80			

Table 6: Values of LC50 and regression equations for methanol roots extract of Morinda citrifolia (L.)

Sample	LC_{50} (µg/ml)	Regression equation	R3
Methanol Morinda citrifolia (L.)	34.50	y = 28.078x - 3.18	0.913

DISCUSSION

Cholera is recognized as an ancient diarrheal disease that is a continuous burden to public health for years in the developing and under-developed countries of Asia and Africa, where there are a population influx and poor water supply and basic hygiene. The growing demand for alternative medicine has led scientists to conduct potential research on plant materials. Plants possess many natural compounds with antimicrobial properties, including tannins, alkaloids, flavonoids, and terpenoids [32]. As a result, the herbal source's antibacterial compounds could be an economical and efficient therapeutic to cure and prevent bacterial infections with a minimal side effect.

In Nigeria, cholera is one of the most detrimental diseases that lead to fatality. Based on a recent survey, out of over 200 million populations of our country, at least with such

population a require protection against cholera due to the emergence of rampant antibiotic-resistant of Vibrio species is needed [8]. Therefore, the present work focused on natural compounds from the plant part sources (roots), Morinda citrifolia (L.), which particularly reported for the presence of several tannins and alkaloids [28, 29].

The methanolic roots extract of Morinda citrifolia (L.) confirmed bioactive compounds, including tannins, alkaloids, saponins, flavonoids, phenols, etc., on phytochemical screening present experiments.

In the current experiment, the in-vitro analysis for anti-V. cholerae activities of these plant sources revealed that V. cholerae is susceptible to methanol roots extracts of Morinda citrifolia (L.) that showed a zone of inhibition similar to that of the commercially available antibiotic (meropenem, 10 µg/disk). Our results evidenced that different extracts' degree of hindering bacterial growth was done in a concentration-dependent manner. The minimum concentrations that inhibited the growth of V. cholerae were 2.50 mg/ml and 10.00 mg/ml for the methanol roots extracts of Morinda citrifolia (L.).

Hence, our study indicated that the organism is more susceptible to methanol roots extracts of Morinda citrifolia (L.) at Concentrations 8 and 10 mg/ml. Besides, the magnitude of the zone of inhibition for methanol extract of Morinda citrifolia (L.) at 13.89±1.73a(**)b(η) was higher than that of gentamicin (12.94 ± 1.66). Therefore, our work suggests that the existing tannins, which reported to hold antimicrobial properties in the methanol roots extracts of Morinda citrifolia (L.) are much potent against V.cholerae.

The cytotoxic potential of all samples was less than vincristine sulphate, indicating that both plants' crude extracts are safe for ethnopharmacological use with low toxicity. Nevertheless, the highest mortality rate and lowest LC50 value was found with methanol roots extracts of Morinda citrifolia (L.). indicating a better cytotoxic profile than any other samples. Moreover, previous studies confirmed that the leaves of methanol leafs extracts of Morinda citrifolia (L.).have weak cytotoxic properties [25], while the fruits exhibited significant cytotoxicity in human cancer cell lines [34].

The toxicity effect of crude plant extract is assessed based on LC50 value where a value less than 1000 µg/ml is considered toxic, confirming the existence of bioactive compounds [35, 36]. Thus, our investigation suggests that methanol roots extracts of Morinda citrifolia (L.).contain active or potent compounds that may exert significant pharmacological activities.

CONCLUSION

In conclusion, our study confirmed that the methanol roots extracts of Morinda citrifolia (L.). have potential anti-V. cholerae activities, with mild toxicity, due to the presence of reported tannins and alkaloids. This indicates that the plant materials, methanol roots extracts of Morinda citrifolia (L.).may be used as reliable sources of herbal remedy against cholera disease.

Competing interests

The authors declare that they have no competing interests

REFERENCES

1. Nishibori T, de Vries GC, Rahardjo D, Wasito EB, De I, Kinoshita S, et al. Phenotypic and genotypic characterization of Vibrio cholerae clinically isolated in Surabaya, Indonesia. Jpn J Infect Dis. 2011;64(1):7–12.
2. Chakraborty S. Plant derived extracts and respective compounds against major life-threatening water contaminant bacteria Vibrio cholerae: a comprehensive mini review. Int Res J Environ Sci. 2015;4(7):96–105.
3. Aminzare M, Hashemi M, Abbasi Z, Mohseni M, Amiri E. Vibriosis phytotherapy: a review on the most important world medicinal plants effective on Vibrio spp. J App Pharm Sci. 2018;8(01):170–7.
4. Reidl J, Klose KE. Vibrio cholerae and cholera: out of the water and into the host. FEMS Microbiol Rev. 2002;26(2):125–39. https://doi.org/10.1111/j.1574-6976.2002.tb00605.x.
5. Harris JB, Larocque RC, Qadri F, Ryan ET, Calderwood SB. Cholera. Lancet. 2012;379(9835):2466–76. https://doi.org/10.1016/S0140-6736(12)60436-X.
6. Colombara DV, Cowgill KD, Faruque AS. Risk factors for severe cholera among children under five in rural and urban Bangladesh, 2000–2008: a hospital-based surveillance study. PLoS One. 2013;8(1):e54395 https://doi.org/10.1371/journal.pone.0054395.
7. Cholera annual report 2012. Wkly Epidemiol Rec. 2013;88(31):321–336. 8. Islam MT, Clemens JD, Qadri F. Cholera control and prevention in Bangladesh: an evaluation of the situation and solutions. J Infect Dis. 2018; 218(Suppl 3):S171–2. https://doi.org/10.1093/infdis/jiy470.
9. Ali M, Nelson AR, Lopez AL, Sack DA. Updated global burden of cholera in endemic countries. PLoS Negl Trop Dis. 2015;9(6):e0003832. https://doi.org/10.1371/journal.pntd.0003832.
10. Saha A, Chowdhury MI, Khanam F, Bhuiyan MS, Chowdhury F, Khan AI, et al. Safety and immunogenicity study of a killed bivalent (O1 and O139) whole cell oral cholera vaccine Shanchol, in Bangladeshi adults and children as young as 1 year of age. Vaccine. 2011;29(46):8285–92. https://doi.org/10.101 6/j.vaccine.2011.08.108.

11. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277–83.

12. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother. 2001; 45(4):999–1007. https://doi.org/10.1128/AAC.45.4.999-1007.2001.

13. Baron S, Lesne J, Jouy E, Larvor E, Kempf I, Boncy J, et al. Antimicrobial susceptibility of autochthonous aquatic Vibrio cholerae in Haiti. Front Microbiol. 2016;7:1671. https://doi.org/10.3389/fmicb.2016.01671.

14. Hochhut B, Lotfi Y, Mazel D, Faruque SM, Woodgate R, Waldor MK.Molecular analysis of antibiotic resistance gene clusters in Vibrio cholerae O139 and O1 SXT constins. Antimicrob Agents Chemother. 2001;45(11): 2991–3000. https://doi.org/10.1128/AAC.45.11.2991-3000.2001.

15. Monroe S, Polk R. Antimicrobial use and bacterial resistance. Curr Opin Microbiol. 2000;3(5):496–501. https://doi.org/10.1016/S1369-5274(00)00129-6.

16. Zhang XY, Han XX, Chen XL, Dang HY, Xie BB, Qin QL, et al. Diversity of cultivable protease-producing bacteria in sediments of Jiaozhou Bay, China. Front Microbiol. 2015;6:1021.

17. Aminzare M, Aliakbarlu J, Tajik H. The effect of Cinnamomum zeylanicum essential oil on chemical characteristics of Lyoner-type sausage during refrigerated storage. Vet Res Forum. 2015;6(1):31–9.

18. Tabrah FL, Eveleth BM. Evaluation of the effectiveness of ancient Hawaiian medicine. Hawaii Med J 1966; 25: 223-30.

19. Krauss B. Plants in Hawaiian culture. Honolulu: University of Hawaii Press; 1993. p103, p252.

20. Whistler W. Tongan herbal medicine. Isle Botanica, Honolulu, Hawaii, 1992. p 89-90.

21. Bruggnecate JT. Native plants can heal your wounds. Honolulu Star-Bulletin Local News 1992 Feb 2.

22. Solomon N. The tropical fruit with 101 medicinal uses, NONI juice. 2nd ed. Woodland Publishing; 1999.

23. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol. 2005;4(7):685–8. https://doi.org/10.5897/AJB2005.000-3127.

24. Yadav M, Chatterji S, Gupta SK, Watal G. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm. 2014;6(5):539–42.

25. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: a review. Int Pharm Sci. 2011;1(1):98–106.

26. Bayer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol. 1966;45(4):493–6. https://doi.org/10.1093/ajcp/45.4_ts.493.

27. Lino A, Deogracious O. The in-vitro antibacterial activity of Annona senegalensis, Securidacca longipendiculata and Steganotaenia araliacea- Ugandan medicinal plants. Afr Health Sci. 2006;6(1):31–5. https://doi.org/10. 5555/afhs.2006.6.1.31.

28. Bauer AW, Kirby QMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4):493–6. https://doi.org/10.1093/ajcp/45.4_ts.493.

29. Konan NA, Bacchi EM, Lincopan N, Varela SD, Varanda EA. Acute, subacute toxicity and genotoxic effect of a hydroethanolic extract of the cashew (Anacardium occidentale L.). J Ethnopharmacol. 2007;110(1):30–8. https://doi.org/10.1016/j.jep.2006.08.033.

30. Badakhshan MP, Sasidharan S, Rameshwar NJ, Ramanathan S. A comparative study: antimicrobial activity of methanol extracts of Lantana camara various parts. Phcog Res. 2009;1(6):348–51.

31. Pisutthanan S, Plianbangchang P, Pisutthanan N, Ruanruay S, Muanrit O.Brine shrimp lethality activity of Thai medicinal plants in the family Meliaceae. Naresuan Univ J Sci Technol (NUJST). 2013;12(2):13–8.

40. Asaduzzaman M, Rana MS, Hasan SR, Islam R, Das N. Phytochemical and antioxidant investigation of barringtonia acutangula (L.). Eur J Med Plants. 2015;8(4):21:231–8.

32. Lu B, Zhou H, Li D, Li F, Zhu F, Cui Y, et al. The first case of bacteraemia due to non-O1/non-O139 Vibrio cholerae in a type 2 diabetes mellitus patient in mainland China. Int J Infect Dis. 2014;25:116–8. https://doi.org/10.1016/j.ijid.2 014.04.015.

28. Princy KR, Sripathi R, Dharani J, Ravi S. Molecular docking studies of alkaloids from Desmodium triflorum against bacterial proteins. J Pharm Sci Res. 2017; 9(10):1882–5.

29. Burapadaja S, Bunchoo A. Antimicrobial activity of tannins from Terminalia citrina. Planta Med. 1995;61(04):365–6. https://doi.org/10.1055/s-2006-958103.

33. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999;12(4):564–82. https://doi.org/10.1128/CMR.12.4.564.

34. Sangthong P, Nittayajaiprom W, Chancharunee S, Wipatanawin A, Wanasawas P, Chulasiri M. Mutagenicity, antimutagenicity and tyrosinase inhibition activity of hydroglycol extracts from Terminalia chebula Retzius, Terminalia bellerica Roxb and Rafflesia kerrii Meijer. Int J Phytomedicine. 2014;6(1):93–102.

35. Lai SC, Ho YL, Huang SC, Huang TH, Lai ZR, Wu CR, et al. Antioxidant and antiproliferative activities of Desmodium triflorum (L.) DC. Am J Chin Med. 2010;38(02):329–42. https://doi.org/10.1142/S0192415X10007889.

36. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DJ, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 1982;45(5):31–4. https://doi.org/10.1055/s-2007-971236.