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In vitro ethno-toxicology of *Hunteria umbellata* methanol seed and stem extracts against *Vibrio parahaemolyticus* and its molecular implication on swarming and adhesion genes

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Abstract

Background *Hunteria umbellatta* (HU) is a predominant plant with a share of therapeutic properties against various human diseases with no scientific report of its in vitro ethno-toxicology coupled with molecular implication in the literature. This study, therefore, evaluated in vitro toxicological activity and quantification of expression of specific swarming gene, lateral flagellar (Laf A) and adhesion gene (ExsE) of *Vibrio parahaemolyticus* (VP) treated with methanol seed and stem extracts of HU at 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625 µg/µL concentrations.

Results The toxicological results of all the eight tested concentrations from as low as 3.90625 µg/µL to as high as 500 µg/µL of HU seed and stem extracts revealed significantly ($p < 0.05$) uninhibited bacterial growth in a dose-dependent manner compared to controls (positive and negative). The quantity of Laf A and ExsE genes' expressions in VP was significantly higher ($p < 0.05$) at 500 µg/µL of HU seed and stem compared to control while at 125 µg/µL of the same extract (seed and stem) showed significantly lowered ($p < 0.05$) expression of swarming and adhesion genes in VP relative to control. Comparative to control, adhesion gene (ExsE) expression in VP significantly increased ($p < 0.05$) at 250 µg/µL of HU seed and stem extracts.

Conclusions The results obtained suggest toxicity at varying concentrations and higher concentration dosing of HU seed and stem is harmful as it could lead to increased expression of the gene colonization factor of VP as a major contributory agent of gastroenteritis.

Keywords *Hunteria umbellata*, Ethno-toxicology, Laf A, ExsE, Gastroenteritis

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Background

Medicinal plants are globally valuable sources of new drugs due to the rising request for natural health products and herbal medications. Africa is not left out in the quest for different parts of medicinal plants as various parts of the *Hunteria umbellata* (HU) plant have been explored to cure human diseases. *H. umbellata* is a member of Apocynaceae plant family frequently available in many sub-Saharan African countries including Nigeria. It is a small tree with dense hard texture leaves, dark brown stems, abundant flowers and large fruits bearing seeds (Fadahunsi et al. 2021). Many disease conditions are treated with almost all the parts of HU plant in traditional medicine without satisfactory validation of dosages and safety. Hence, there is need for caution in the misinformation on adequate doses and uses of *H. umbellata* by users because there has been an established connection between toxicological and phyto-therapeutical potentials of medicinal plants in literature (Eddouks et al. 2019). This is because microbial contamination is frequently common in herbal medicines and some plant-based medicines have been shown to induce toxicity due to interactions with normal body cells and subsequently pose high health risks (Okaiyeto and Oguntibeju 2021). Therefore, toxicological research on any medicinal plant should be extensively conducted (Kahaliw et al. 2018). Several in vivo studies have reported pharmacological and toxicological properties of HU to prove its efficacy and safety. The stem extract at 150 and 300 mg/kg has been reported to significantly ($p < 0.05$) reduced the number of writhes and paw licking times in animal models despite associated increased pain threshold in writhing assay, paw licking and hotplate tests from the same study (Edosuyi et al. 2017). Anti-hypertensive activity of HU seed (100 to 400 mg/kg body weight) in rodent has been elucidated by Ajiboye et al. (2017) while a nontoxic effect of methanol extract of the seed of HU at 250, 500 and 1000 mg/kg/day on the reproductive organs and functions in masculine and feminine rats was established in the study of Oseyomon and Ilodigwe (2021). Also, Momodu et al. (2014) demonstrated the anti-hyperglycemic potential of 250 mg/kg *H. umbellata* aqueous seed extract in rats. However, very few in vitro studies reported antibacterial activity of HU stem bark extract but without molecular consequence of the extract. In vitro study of Peter et al. (2020) established least inhibitory effect of different HU plant parts (Stem, Seed and Leaf) when compared with *Moringa oleifera* and *Azadirachta indica* in solitary and synergy with three different antibiotics (Azithromycin, Clindamycin and Vancomycin) against Methicillin–Resistant *Staphylococcus aureus* strains. Also, the antibiotic and therapeutic properties of HU seed extracts against some selected clinical isolates (*Staphylococcus sp., P. aeruginosa, Micrococcus sp., Klebsiella sp., Proteus sp., Bacillus sp., Candida albicans, S. cerevisiae and Escherichia coli*) have been recognized in recent study (Udinyiwe and Aghedo 2022) with the exception of *Vibrio parahaemolyticus*. *V. parahaemolyticus* (VP) is an important gram-negative pathogenic bacterium in estuarine, marine and coastal environs that is usually available in a free-swimming state (Letchumanan et al. 2014). Its clinical significance has been documented as the fundamental agent of human acute gastroenteritis, wound and ear infections (Zhang and Orth 2013). Thus, elucidation of HU stem and seed at the same doses in VP bacterial activity will advance the knowledge of medicinal use for the treatment of abdominal discomfort and stomach ache. Furthermore, identification, evaluation and quantification of specific cluster of virulence genes such as Laf A and ExsE that help pathogenic bacteria to thrive and colonize human host will increase our understanding of modeling genetic markers to profile and adequately predict the toxicity of many medicinal plants. The role of these acquired virulence genes in bacteria through horizontal gene transfer (HGT) in evolution and ability to cause gastroenteritis as replicated in nosocomial infection has been well documented to understand their pathogenicity (Bayode et al. 2020). Pathogenicity determinants of VP have been linked to Laf A gene and ExsE protein. ExsE, a highly charged protein, is secreted in a T3SS-dependent manner, required for the initial adhesion of VP while expression of Laf A gene is responsible for lateral flagella production needed for swarming motility of VP in a human host (Zhang et al. 2023). Consequently, the addition of the high sensitivity of the evolving molecular techniques (e.g., qPCR) will make it possible to detect responses at low, medium and high dosages of any understudied plant extract or compound. However, the in vitro toxicity of vital parts of the HU plant, such as the seed and stem in *V. parahaemolyticus* as a gastro-enteritic agent, and especially the molecular effect on its swarming (Laf A) and adhesion (ExsE) genes, is scarcely described in the literature. As a result, this study was designed to specifically provide scientific evidence on in vitro ethno-toxicity of HU seed and stem and their effect on genes' expression in the causative agent of gastroenteritis (*V. parahaemolyticus*). This is to establish or nullify the efficacy and health hazards accompanying the consumption of the most harnessed (seed) and less harnessed (stem) part of the HU plant as a source of drug or disease management. In addition, to lay the groundwork for potential medicinal plant-resistance mechanisms of action underlying a toxic response in human highly infectious diseases.

Methods

Plant extract preparation

Hunteria umbellata's fresh green leaves and fruits were bought in February 2023 from a resident marketplace in Dopemu, Lagos State, Nigeria (6° 07' N to 7° 00' N latitude and 3° 43' E to 4° 00' E longitude). The HU plant samples were identified, authenticated and assigned voucher number LUH 8995 and LUH 8996, respectively, in the Department of Botany, Faculty of Science, University of Lagos. The plant samples were further deposited in the herbarium of the same university. The stem of HU was separated from the leaves while the seeds were harvested from the fruits, washed with clean tap water, and sun-dried separately for 7 days to protect the bioactive components of the plant. The dried seed and stem samples were coarsely milled separately using an electronic milling machine grinder, Lab. Mill, Serial No. 4745, Christy and Norris Ltd. The plant extraction was carried out via a modified method of Udinyiwe and Aghedo (2022). The ground HU stem (73 g) and seed (70 g) were macerated in 400 ml of 99.8% methanol for 72 h separately. Each homogenate was filtered at the end of the extraction using a muslin cloth and cotton wool to remove the filtrate from the residue. Each filtrate was oven-dried at 40 °C, kept in a glass bottle for further studies.

Clinical bacterial isolate and antibacterial assay

A gram-negative, halophilic human pathogenic bacterium, *Vibrio parahaemolyticus* (VP) strain ECN601, was used for this study. The clinical isolate was sourced from the Department of Microbiology, Nigerian Institute of Medical Research (NIMR), Lagos State. The spread plate method was employed to sub culture the isolate on nutrient agar (Thiosulphate citrate bile salt) at 37 °C for 24 h to acquire a pure culture. Nutrient broth (100 µL) containing inoculated bacteria at the 0.5 MC Farland standard equivalent of 1.5×108 colony forming units (CFU) was prepared and added to each well of the microplate (column). Each (22 g) methanol seed and stem extract of HU was dissolved in 2.3% dimethyl sulfoxide (DMSO), which would not inhibit the growth of bacteria (Verheijen et al. 2019). A working concentration of 1 mg/ml of the susceptible antibiotic, tetracycline, was also prepared by dissolving 1 mg of tetracycline powder in 1 ml of sterile water as the reference antibiotic for the microbial experiment. The broth microdilution method described by Lallemand et al. (2016) was employed with a two-fold serial dilution of HU methanol stem extract, resulting in eight (8) final concentrations of 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 and 3.90625 µg/µL from columns 3 to 10, respectively. Column 1 contained DMSO (2.3%), and the test bacteria were inoculated in nutrient broth to serve as the negative control, while 10 µL of tetracycline was added to column 2 as a standard positive control in addition to the nutrient broth and VP (Yano et al. 2014). However, column 11 represented the blank with only 200 µL sterile nutrient broth, and other wells of the microplate were made up to 200 µL by adding 100 µL nutrient broth containing inoculated bacteria at the 0.5 MC Farland standard, which equals 1.5×108 CFU. The microplate was incubated at 37 °C on a shaker for 24 h. The optical density (OD) was also read at 600 nm using a Varioskan LUX Multimode microplate reader (version 1.00.38), before and after incubation to determine the growth or inhibition rate at the end of the experiment. Resazurin reagent is a reagent that has an original blue colour that changes to pink during reaction with oxygen to indicate viable microbes. Thirty microliters (30 µL) of 0.8 mg/ml resazurin were prepared by dissolving 0.8 mg in 1 ml of water and added to each well of the microplate after 24 h of incubation. This was incubated for another 2–4 h to visibly mark the point of inhibition or to ascertain the minimum inhibitory concentration of the plant extract.

Isolation and purification of genomic RNA

A spin column method according to Shi et al. (2018) was employed. Genomic single-stranded ribonucleic acid (RNA) was isolated using the silica-gel adsorption method from bacteria treated with a high dose (500 µg/µL), medium dose (250 µg/µL), low dose (125 µg/µL) of HU methanol stem extract, a negative control (DMSO-treated), and a positive control (tetracycline-treated) from the antimicrobial assay using the NIMR Biotech RNA purification kit according to the manufacturer's instructions (NIMR Biotech, Lagos, Nigeria). Generally, the processes involved lysis of the cell, incubation, precipitation of RNA, washing of the cell debris and impurities, elution of RNA using the spin column-based method, and storage at − 20°C in the freezer till further use.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) was carried out to convert the extracted RNA to complementary DNA (cDNA) using Solis Biodyne firescript cDNA synthesis mix with Oligo (DT) and random primers according to manufacturer's description. To make a 10 µl reaction mixture, 6.8 l of molecular-grade water, 2 µl of master mix, 0.1 µl of forward and reverse primers, and 1 µl of RNA template were added. The primer annealing occurred at 25 °C for 8 min, at 50 °C for 30 min, and enzyme inactivation at 85 °C for 5 min in a conventional thermal cycler (Peltier Thermal Cycler 200, MJ Research). The product (cDNA) was preserved in the freezer at − 20 °C until usage.

Amplification and quantification of Laf A and ExsE genes

Quantitative real-time polymerase chain reaction (QPCR) was employed to quantify the expression of lateral flagellar (Laf A) and ExsE genes of *Vibrio parahaemolyticus*, which are used to move in groups and adhere to the host's cell. The reaction was carried out using forward (F) and reverse (R) primer pair of qLafA (F: 5`CGC AGC TAT CAC TGAC GGT A 3`; R: 5`TCC ATG ATA CGG CCT TTA GC 3`) and qExsE (F—5` AAC GTT TCA AGG TCG CAA AG 3`; R- 5` TAC CTT TCG CTT CGA GCA AT 3`) as previously described by Erwin et al. (2012) and synthesized by the NIMR-MTN Oligo Synthesis Laboratory. The quantitative PCR was carried out using Solis Biodyne 5×HOT Firepol Evagreen® qPCR supermix, according to the manufacturer's instructions, to quantify the gene of colonization using the designed primers by the Department of Biochemistry, NIMR. The supermix is a ready-to-use mix containing chemically modified FirePol DNA polymerase that enables hot starts, an optimized PCR buffer, MgCl2, dUTPs, dNTPs, and EvaGreen dye. The reaction was performed in a 10 µl reaction volume containing 2µL Evagreen qPCR supermix(5×), 0.1 µl of 100 pmol of each set of primers (forward and reverse), 2 µl of the cDNA template from the first RT-PCR reaction, and 5.8 µl double-distilled nuclease-free water. The amplification was done at an initial denaturation temperature of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s. The final elongation was done at 72 °C for 5 min. The temperature was further raised to 95 °C for 30 s and 65 °C for 30 s, and the melt curve was performed at 0.5 °C increments of 65–95 °C for every 1 s before the cycle was terminated in a BioRad CFX96 Deepwell Real-time system.

Data analysis

Microbial and Ct values (threshold frequency) generated at the end of the reaction using the formula: $2^{\Delta\Delta CT}$ were subjected to analysis using IBM SPSS statistics version 26 (SPSS Inc. PASW Statistics for Windows, Chicago: SPSS Inc.), and Excel 2016 version (Microsoft). All values were presented as mean ± SEM (standard error mean) and analyzed by one way ANOVA followed by a post hoc test of LSD (least significance difference) to compare the mean difference. Significance was set at 95% confidence interval to indicate differences among compared groups.

Results

Bactericidal activity of HU seed and stem extracts against *V. parahaemolyticus*

The results obtained from monitoring anti-vibrio activities of the eight tested concentrations of HU seed and stem respectively are shown in Figs. 1 and 2. Optical densities at 600 nm of each extract were measured before and after incubation of five readings ($n=5$) to display the growth of cells. All the eight concentrations of HU seed and stem (500 µg/µL being the highest concentration) examined are presented in a graphical format in Figs. 1 and 2. None of the eight tested extracts shows anti-vibrio property as significant ($p < 0.05$) increase in the growth of cells (VP) compared to controls (positive and negative) were recorded. Tetracycline (a positive control) significantly ($p < 0.05$) inhibited the growth of *V. parahaemolyticus* isolate relative to test groups after incubation as shown below in Figs. 1and 2.

Amplification and quantification of genes' expression (swarming & adhesion genes) in VP exposed to three selected doses of HU methanol seed and stem extracts

The amplification curves of Laf A and ExsE genes in VP exposed to HU methanol seed and stem extracts are presented in Figs. 3 and 4 respectively. The curves are indicative of the binding of DNA to SYBR green fluorescent dye present in the master mix which started after the 30th cycles in Laf A and 20th cycle in ExsE of real-time PCR. The result of swarming and adhesion genes' quantification of HU methanol seed and stem extracts is displayed in Figs. 5 and 6 below. Methanol seed extract of HU at 250 µg/µL recorded highest significant ($p < 0.05$) rise in the quantity of ExsE gene expression in VP relative to other concentrations and control (Fig. 5). The quantity of Laf A and ExsE genes' expressions in VP was significantly higher ($p < 0.05$) at 500 µg/µL of HU seed compared to control whereas at 125 µg/µL of the same extract exhibited significantly lowered ($p < 0.05$) expression of swarming and adhesion genes in VP comparative to control. Expression of LafA gene was suppressed at 250 µg/µL and 125 µg/µL out of the three examined methanol stem concentrations compared to control. A significant ($p \leq 0.05$) increase in the expression of ExsE and LafA genes was highest at 500 µg/µL of *H. umbellata* methanol stem extract (Fig. 6). Only 125 µg/µL of the extract suppressed the expression of ExsE gene while increased expression was observed at 500 µg/µL and 250 µg/µL. Differences between compared groups were considered significant for $p \leq 0.05$.

Discussions

A plethora of reports in the literature have authenticated the efficacy of African medicinal plants for the treatment of diverse human infections with scarce molecular toxicological evaluation to ascertain their safety to public health. Thus, further rigorous study on traditional medicinal plants has been instigated by WHO to address several gaps that will substantiate their potency and safety usage (WHO 2012). Hence, this call necessitates

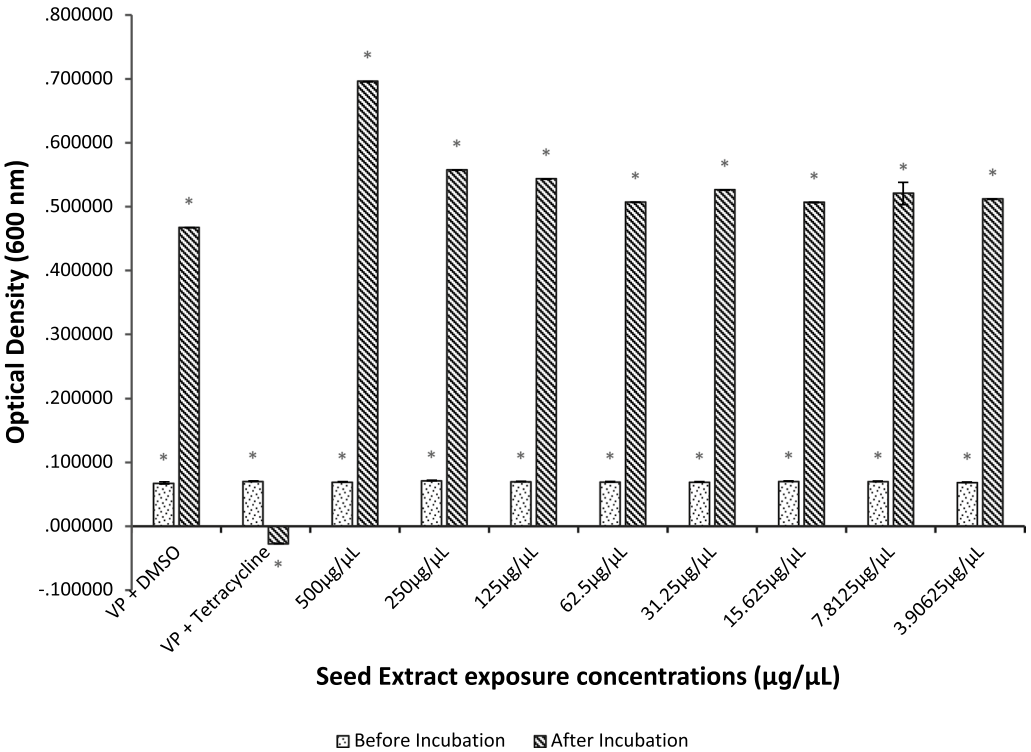

Fig. 1 Growth of VP exposed to graded doses of HU methanol seed extract against antibiotic (Tetracycline). * Error bars represent standard deviation ($n=5$); * = means are significantly different among the positive (VP + Tetracycline) control, negative (VP + DMSO) control and HU exposure concentrations at $p<0.05$ for VP growth. *VP Vibrio parahemolyticus, DMSO* Dimethylsulfoxide

investigation of molecular implication of *H. umbellata* plant at gene level, as part of an extensive study to ethno-toxicology of medicinal plant. *H. umbellata* plant is one of the plants that has been screened for their antimicrobial effects which has been reported to possess antibacterial activity for the treatment of gastroenteritis related illnesses (cholera, dysentery and diarrhea) in which *Vibrio parahaemolyticus* is a pathogen (Igbe et al. 2010; Udinyiwe et al. 2022). Surprisingly and conversely, this study revealed growth enhancement of a gram-negative bacteria (*V. parahaemolyticus*) when exposed to HU, indicative of supporting infection/disease of the stomach. This study shows uninhibited growth of VP in all the eight tested concentrations from 3.90625 µg/ µl to 500 µg/ µl of the HU seed and stem extracts investigated. This means that the consumption of HU for the treatment of gastro enteritis as claimed in folkloric medicine may pose dangers to the health. However, their claims could be as a result of the presence of some constituents in the leaf extract which carried out a synergistic effect with the stem (Falodun et al. 2006) but not the effect of the stem bark or seed alone. On the contrary, the result in Fig. 1 above indicates that the microbial growth increases with concentration of the extract (dose-dependent manner). This suggests that the use and overuse of methanol *Hunteria umbellata* stem extract provides room for microbial infection dangerous to the health (in this case gastro enteritis or diarrhea). Thus, our result disagrees with the earlier reported antibacterial activity of *Hunteria umbellata* by Udinyiwe et al. (2022) which stated that the stem extract *of H.umbellata* was active against gram positive and gram negative bacteria. However, this study is in accordance with the result of Igbe et al. (2009) where HU extract concentrations (up to 75%w/v) failed to cause inhibition of clinical isolates (*S. aureus*, *E. coli*, *K. pnuemoniae* and *P. aeruginosa)* when compared to the inhibitory effect of the standard drug (Ciprofloxacin).

Virulence gene in pathogenic organisms such as bacteria are critical for causing diseases when colonization of the infected host is successful (Upadhyay et al. 2017). A variety of virulence factors acquired through horizontal gene transfer mechanisms such as conjugation, transformation, and transduction are employed in VP to initiate infection in the host (Bayode et al. 2020). These include swarming behaviour over solid surfaces or during host colonization and adhesion character in the host intestine (Letchumanan et al. 2014). In our study, we analysed these selected colonization genes; swarming genes (LafA) and adhesion genes (ExsE) which were slightly expressed variably. Lateral flagellar

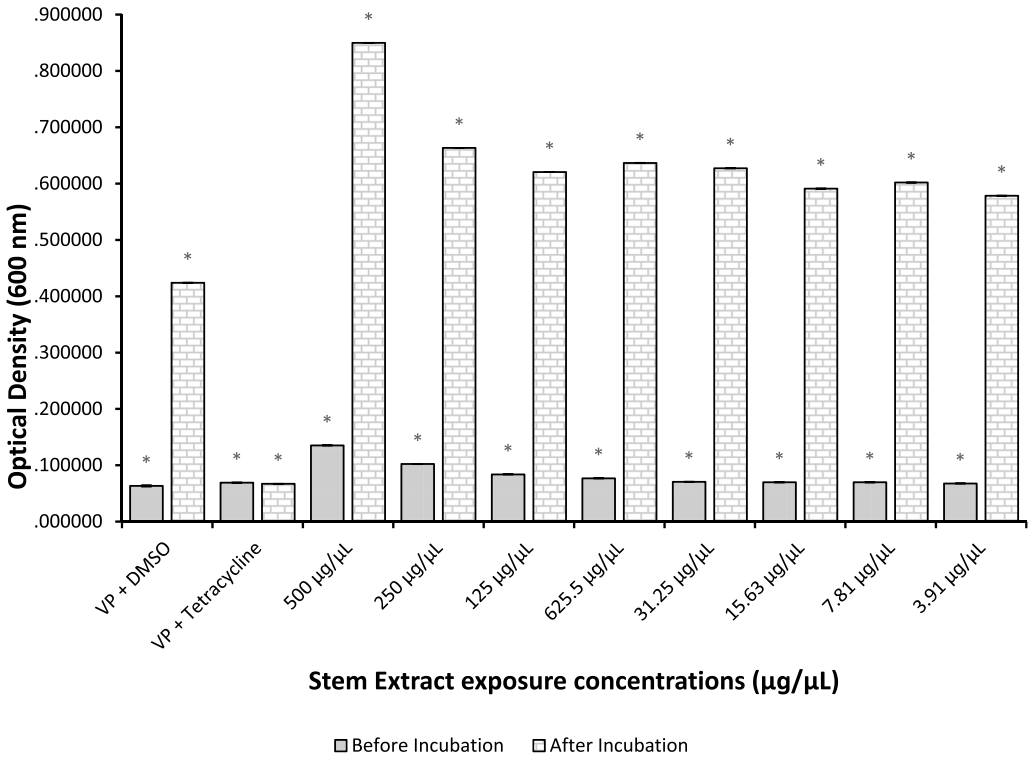

Fig. 2 Growth of VP exposed to graded doses of HU methanol stem extract against antibiotic (Tetracycline). Error bars represent standard deviation ($n=5$); * = means are significantly different among the positive (VP + Tetracycline) control, negative (VP + DMSO) control and HU stem exposure concentrations at $p < 0.05$ for VP growth. *VP Vibrio parahemolyticus, DMSO* Dimethylsulfoxide

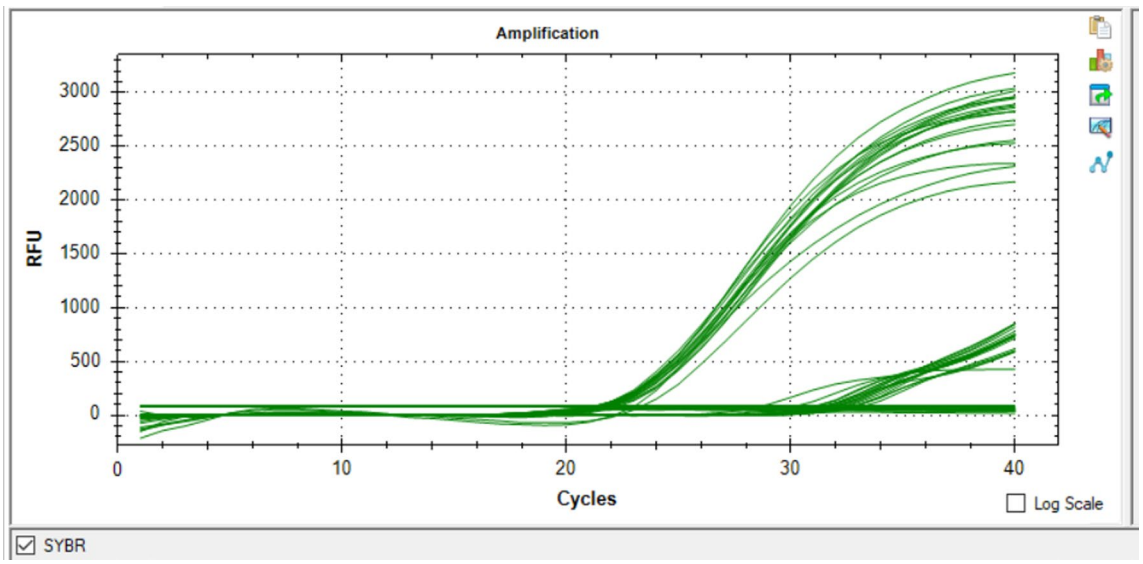

Fig. 3 Amplification curves of Laf A genes of VP treated with HU methanol seed and stem extracts. RFU represent relative fluorescence units

gene was suppressed at moderate (250 µg/µL) and low (125 µg/µL) doses of seed and stem, while adhesion gene was suppressed at 125 µg/µL of HU stem extract. Although, *H. umbellata* failed to inhibit the growth of *V. parahaemolyticus* as shown in Fig. 1 above but further quantification of the expressed colonization genes (LafA and ExsE), at low and moderate concentrations significantly decreased compared to control.

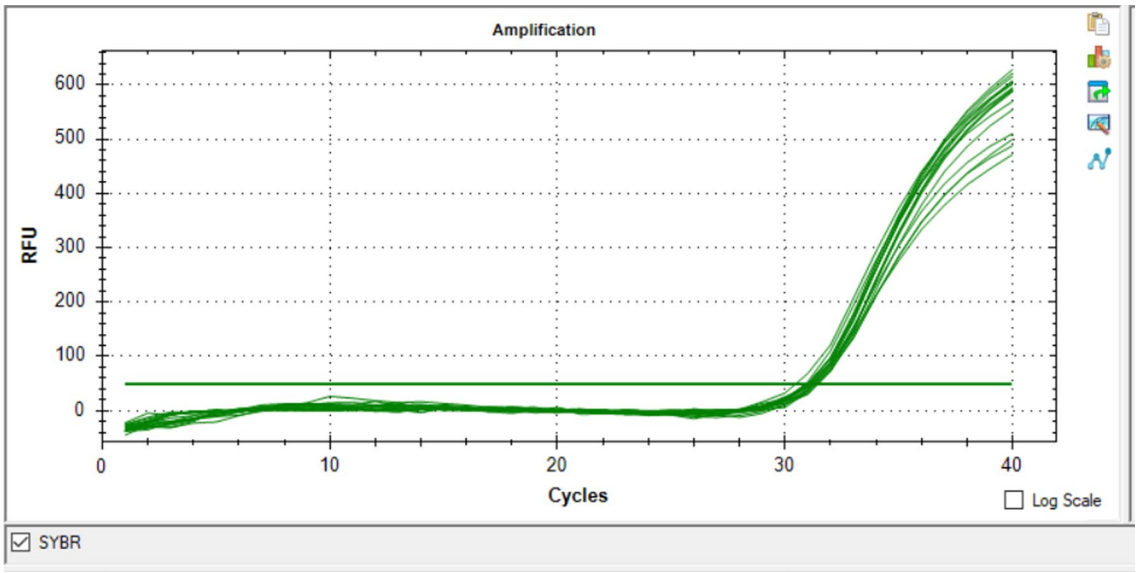

Fig. 4 Amplification curves of ExsE genes of VP treated with HU methanol seed and stem extracts. RFU represent relative fluorescence units

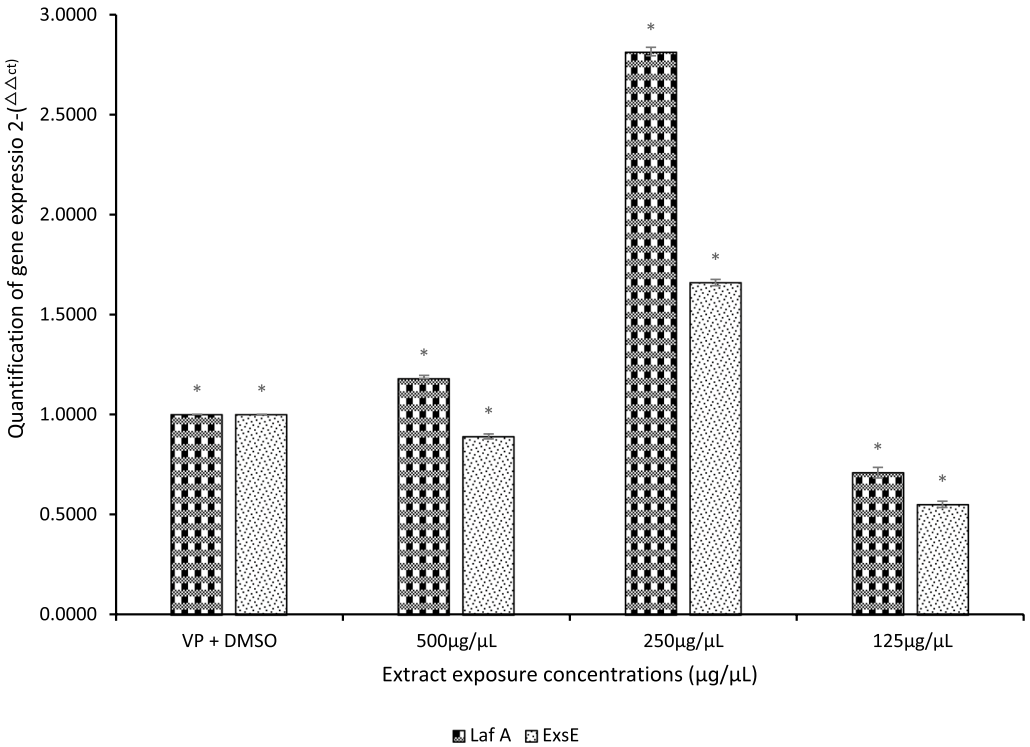

Fig. 5 LafA and ExsE genes' expression quantification in VP treated with HU methanol seed extract. Error bars represent standard deviation ($n = 5$); * = means are significantly different between the VP + DMSO (control) and HU exposure concentrations at $p < 0.05$ for swarming (LafA) and adhesion (ExsE) genes. *VP Vibrio parahemolyticus, DMSO* Dimethylsulfoxide

This result is at par with positive antibacterial activity at low concentrations (< 50 mg/ml) reported in a study of Udinyiwe and Aghedo (2022). The growth of the bacterial cells could naturally be increasing but not causing disease since these two genes were least expressed at lower concentrations (LafA; 250 µg/µL and 125 µg/µL, ExsE; 125 µg/µL). This implies that the extract exerts its antibacterial activity at genetic level

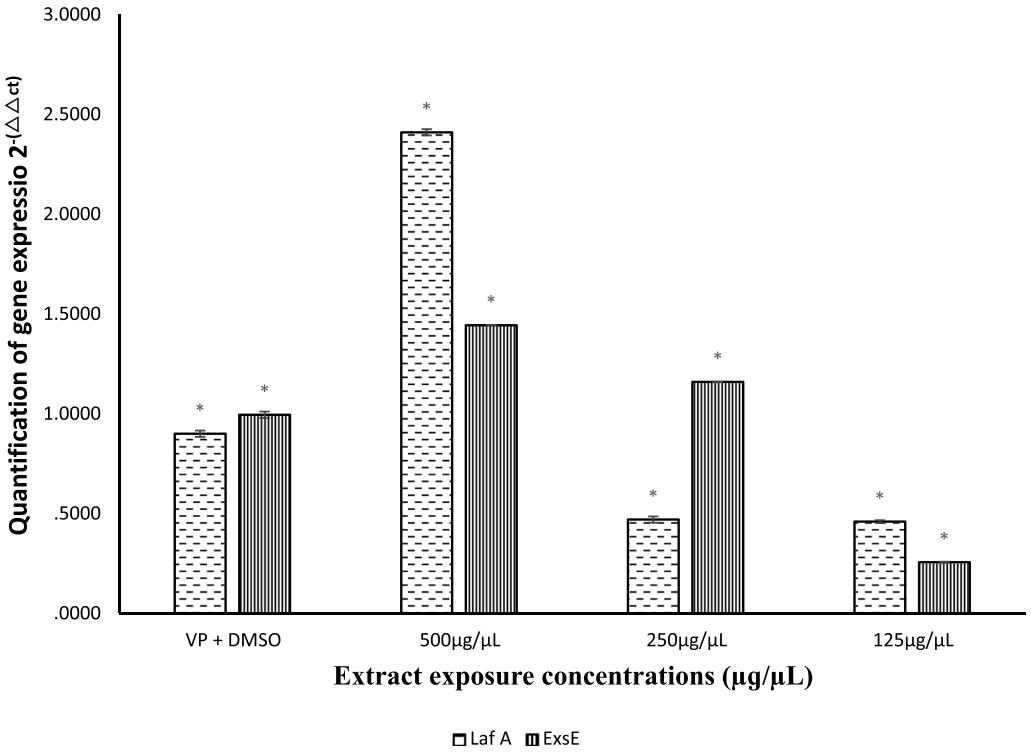

Fig. 6 LafA and ExsE genes' expression quantification in VP treated with HU methanol stem extract. Error bars represent standard deviation ($n = 5$); * = means are significantly different between the VP + DMSO (control) and HU exposure concentrations at $p < 0.05$ for swarming (LafA) and adhesion (ExsE) genes. *VP Vibrio parahemolyticus, DMSO* Dimethylsulfoxide

and is unable to colonize the host using these colonization factors (LafA and ExsE gene) at low concentrations. However, an increase in the expression of both genes were recorded at high dose (500 µg/µL) of HU stem extract. It was reported in the study of Erwin et al. (2012), that ExsE and LafA genes are colonization genes of *V. parahaemolyticus* and deletion of any them will lead to non-functional of the other. Our study aligned with the previous study as it was shown that the ExsE and LafA genes work hand in hand, as they were both expressed invariably the same manner in the seed and stem of HU. Medications such as ampicillin, tetracycline and gentamycin have been used to manage diseases caused by this understudied gastroenteritis-causing bacterium (Bruzzese et al. 2018; Mok et al. 2019). This study affirms the action of tetracycline against *V. parahaemolyticus* as reported in the literature. Nevertheless, our findings from the quantification of the expressed genes responsible for swarming and adhesion of *V. parahaemolyticus* after exposure to HU seed and stem extracts at 125 µg/µL (low dose), 250 µg/µL (moderate dose) and 500 µg/µL (high dose) are scarce in the literature.

Conclusions

This study has been able to show molecular toxicology at gene level in addition to microbial activity of *Hunteria umbellata* methanol seed and stem extracts from low to high doses is detrimental to human health. Therefore, to establish effective use of any part of HU to reduce the risk infection by this bacterium, *V. parahaemolyticus*; employment of molecular methods must be incorporated in the toxicology for the detection of *V. parahaemolyticus* in herbal extracts is very important. Infections caused by multidrug-resistant of *V. parahaemolyticus* strains also pose a substantial risk to human health.

Recommendation

It is strongly recommended that medicinal plants are rigorously investigated with modern molecular approach in addition to in vivo studies to ascertain the safety towards infectious diseases management and drug development. Further elucidation of the bioactive compounds of *H. umbellata* responsible for enhancement of *Vibrio parahaemolyticus* infections is advised to support unsafe use of the extract in the management of gastroenteritis.

Abbreviations

HU	*Hunteria umbellata*
Laf A	Lateral Flagellar
VP	*Vibrio parahaemolyticus*
qPCR	Quantitative polymerase chain reaction
NIMR	Nigerian Institute of Medical Research
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
OD	Optical density
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
cDNA	Complementary deoxyribonucleic acid
DT	Deoxy-thymidine
CT-value	Cycle-threshold value
SEM	Standard error mean
ANOVA	Analysis of variance
LSD	Least significant difference

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Author contributions
TF conceptualized the research, participated in the laboratory work, performed data analysis and drafted the paper. MA and LA were involved in primer selection, laboratory work and data collation. TO, MA and IB were involved in selection of microorganisms, laboratory work, data collection and contributed in writing the manuscript. TA and KA ensured quality assurance, participated in molecular data analysis and revised the manuscript. FO and LA revised and edited the final manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated and analyzed during this study are included in this research article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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