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Antibacterial effect of aqueous cold leaf extract of *Eichhornia crassipes* on *Campylobacter jejuni* NCTC 11168

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Abstract
Extensive and imprudent use of antibiotics has resulted in the alternate increased application of phytoextracts as antimicrobials. One of the grave public health challenges is the emergence of antibiotic resistance among bacterial pathogens on a global scale. This study was undertaken to evaluate the potential of crude aqueous cold leaf extract of *Eichhornia crassipes* (*E. crassipes*) for it's antibacterial effect on the Gram-negative microaerophilic foodborne pathogen, *Campylobacter jejuni. Eichhornia crassipes* (water hyacinth), an aquatic plant of the family Pontederiaceae, has traditionally been used worldwide for phytoremediation of polluted water, as green manure/compost, for biogas production, to suppress weeds and even for preparation of crude medicines. The yield of the crude aqueous extract, phytochemical (secondary metabolites) screening of the aqueous extract, and *in vitro* antibacterial activities against *C. jejuni* NCTC 11168 was assessed. Cold leaf extracts from water hyacinth were tested for the presence of various active chemical constituents *viz.*, steroids, alkaloids, flavanoids, triterpenes, saponins and tannins. The minimum inhibitory concentration using agar well diffusion method which was 25 mg/mL, was determined for evaluating the antimicrobial activity of the extract using Mueller Hinton Agar plates with 5 per cent defibrinated sheep blood. The antibacterial activities were expressed as mean diameters (mm) of inhibition zones and categorized as >15 mm, 10–15 mm, and <10 mm for strong, moderate, and weak, respectively. Qualitative analysis revealed that extracts of the leaves were positive for steroids, alkaloids, tritermines, phenol, saponins, glycosides and flavanoids. It can be concluded that the aqueous leaf extract of *E. crassipes* could be utilised as a potential source of natural antibacterial agent and for the development of therapeutic antibiotics against many Gram negative organisms like Campylobacter.

Keywords: *Eichhornia crassipes*, leaf extract, *Campylobacter jejuni* NCTC 11168, antibacterial, MIC

Introduction
Indiscriminate use of antimicrobial agents has resulted in the emergence of the antimicrobial resistance, worldwide with plant-based, traditional medicinal systems taking over, in recent years. The biologically active compounds in traditional plants provide an alternative strategy, and thus have the potential to be developed into chemotherapeutic agents, and as of recent times, an increase in the use of herbal medicines has also been observed in the developing world. *Eichhornia crassipes* (Water hyacinth), which is a freshwater aquatic plant of the family Pontederiaceae and basically a native to Brazil and the equatorial region, is a plant with potential antimicrobial properties. The plant has been used worldwide as green manure/compost, for biofuel production (Bhattacharya and Kumar, 2010) [1] treatment of polluted water on account of the natural ability of the roots to absorb pollutants, especially toxic chemicals like mercury, lead and strontium 90, as well as it's use for preparation of crude medicines. And recently has been shown to have significant in-vitro antibacterial activity against Gram-positive and Gram-negative organisms. Various crude extracts using different solvent systems of the plants have been screened for the antimicrobial property, but limited studies have been performed on the aqueous extracts.
Campylobacter, a major cause of human gastroenteritis, worldwide are normal bacterial flora in poultry, including wild birds and animals like pigs and cattle. Though consumption of contaminated poultry is the major source of infection, drinking contaminated water or unpasteurized milk and contact with farm animals also lead to infection. Thermophilic *Campylobacter* spp., especially, *Campylobacter jejuni* (*C. jejuni*) is of considerable public health concern, worldwide, causing bacterial gastroenteritis in humans sometimes with noticeable morbidity and mortality rates (Chuma *et al.*, 2016 and Shams *et al.*, 2017) [2, 3].

Campylobacteriosis led to 37,600 deaths per year worldwide (WHO, 2015) [4]. Though a self-limiting disease, serious long-term consequences include peripheral neuropathies like Guillain-Barre Syndrome, Miller Fisher Syndrome, and functional bowel diseases, like Irritable Bowel Syndrome (Khoshbakht *et al.,* 2014 and Ronner *et al.,* 2004) [5, 6]. The effect of antibacterial activities of the leaf extract on *Campylobacter* spp. has not been tried, and studies on other organisms are also scanty. Therefore, the main objective of the study was to evaluate the antibacterial activity of the aqueous cold crude leaf extract of *E. crassipes* against *C. jejuni* NCTC 11168. by agar diffusion method and to determine the minimum inhibitory concentration (MIC).

This study was envisaged to determine the yield of crude aqueous leaf extract, the phytochemical (secondary metabolites) screening of the leaf extract and to investigate the in-vitro antibacterial activity against *C. jejuni*.

Materials and Methods

Collection and Authentication of Plant

The whole plant of water hyacinth (*Eichhornia crassipes*) was collected from a water body in Irinjalakuda, Thrissur district as shown in figure 1.

Fig 1: *E. crassipes* plant

The plant, *Eichhornia crassipes* (Mart.) Solms, Pontederiaceae, was collected from a freshwater running stream in Irinjalakuda, Thrissur, Kerala and identified as *Eichhornia crassipes* (Mart.) Solms by the Raw Material Herbareum and Museum, Delhi (RHMD), Council of Scientific and Industrial Research - National Institute of Science Communication and Information Resources, New Delhi, India. A voucher specimen (No. NISCAIR/RHMD/Consult/2020/3618-19) was deposited at the Raw Material Herbareum and Museum (RHMD), Delhi.

The upper part of the plant including leaf, stem and flowers, without the root portion form the leaf extract. The whole plants of *E. crassipes* were washed three-four times with tap water and shade dried for three weeks. The upper plant parts were pulverised, using an electrical pulveriser. The preparation of aqueous extracts was done as per the procedure of Harborne (1991) [7], with slight modifications.

Preparation of aqueous cold extract

One hundred grams of coarse powder of upper part of the plant (*E. crassipes*) was weighed and placed in a 500 mL conical flask with 200 mL of distilled water. Flasks were placed on an Orbital rotary shaker for three days at 100 rpm. Each day the extract was filtered and transferred into another flask. The flask with powder extract was again replenished with 150 mL distilled water and the filtering process repeated. Finally, the quantity was reduced from approximately 200 mL filtrate to around 20 mL and then dried in a rotary evaporator. The extract was then freeze dried (Freeze Operon 7707, Korea) at - 37^{o}C for 8 h. to remove water and reduced to a powder. The percentage yield of plant leaves and stem crude extract was calculated according to Joshi and Kaur (2013) [8].

Percentage yield = Extract weight(g) / Dry weight of plant material(g) x 100%

The extract powder was stored in an air-tight container and kept refrigerated at 4°C to use for analysis.

Preliminary Phytochemical screening of the leaf extract

Qualitative analysis of the secondary metabolites of aqueous leaf extract of *E. crassipes* was carried out for evaluating the presence of various active chemical constituents *viz.*, steroids, alkaloids, flavanoids, triterpenes, saponins and tannins, as per the procedure described by Harborne (1991) [7].

Test for Steroids and Triterpene:

- Salkowski Test: About five milligram of extract was mixed with three millilitre of chloroform. It was then shaken with three millilitre of concentrated sulphuric acid. The development of red colour revealed the presence of steroids. The development of yellow colour in the lower layer on standing, indicated the presence of triterpenes.
- Test for Alkaloids: A 0.5 g of extract was mixed with five millilitre of ammonia and was then extracted with equal volume of chloroform. To this, five millilitre of dilute hydrochloric acid was added and acid layer was obtained.
- Mayer's Test: To one millilitre of acid layer, few drops of Mayer's reagent (1.358 g mercuric chloride dissolved in 60 mL of water which was poured into a solution of five gram of potassium iodide in 10 mL water and made the volume upto 100 mL with distilled water) was added. The development of creamy white precipitate indicated

the presence of alkaloids.
- Wagner's Test: Added few drops of Wagner's reagent (two grams of iodine and six grams of potassium iodide, dissolved in 100 mL of water) to one millilitre of acid extract. Development of reddish brown precipitate indicated the presence of alkaloids.
- Test for phenolic compounds: Five milligram of the extract was mixed in one millilitre of water to which five drops of 10 per cent ferric chloride was added. The development of dark blue colour indicated the presence of phenolic compounds.

Test for Saponins
- Foam Test: Five milligram of extract was shaken with three millilitre of water. The development of foam that persisted for 10 minutes indicated the presence of saponins.

Test for Glycosides
- Sodium Hydroxide test: A small amount of the extract was mixed with 1mL water and six drops of 10 per cent sodium hydroxide solution was added to it. Development of yellow colour indicated the presence of glycosides.

Test for Tannins
- Ferric chloride Test: Two milligram of extract was mixed with three milliliter of one per cent ferric chloride solution. The presence of blue /green/brownish colour revealed the presence of tannins.

Gelatin Test
- A 0.5 gram of extract was mixed with few drops of one per cent solution of gelatin containing 10 per cent sodium chloride. The formation of white precipitate indicated a positive reaction.

Test for Flavanoids
- Ferric chloride test: To two millilitre of alcoholic solution of extract (0.5 g of extract in 10 mL of methanol), a few drops of neutral ferric chloride was added. The formation of green colour indicated the presence of flavanoids.

- Lead acetate Test: To two millilitre of alcoholic solution of extract, a few drops of 10 per cent lead acetate was added. The development of yellow precipitate indicated a positive test.

Preparation of Different Concentrations of the Extracts
The stock solution (leaf aqueous extract of *E. crassipes*) was prepared by reconstituting 1.4 g of each of the extracts in 7 ml of sterile distilled water. Different concentrations (6.25, 12.5, 25, 50, 100, 200 and 250 mg/mL) were then prepared from the stock.

Bacterial Strain
The reference culture *Campylobacter jejuni* NCTC 11168, which is a human pathogenic bacteria, was procured from Culture Collections (Public Health England), England. The bacteria was maintained in Mueller Hinton broth (MHB) at -80C and at time of use, was inoculated onto the selective media, Blood-Free Campylobacter Selectivity (modified Charcoal Cefoperazone Deoxycholate, mCCD) agar media supplemented with cefoperazone, teicoplanin, amphotericin B selective supplement (FD 145) and Campylobacter supplement V (FD 067). The bacterial culture was prepared overnight (24 h) at 42°C in Mueller Hinton broth for the preparation of cell suspensions, which were then adjusted to the turbidity of 0.5 McFarland standards (10^8 CFU/mL), as described by the Clinical Laboratory Standard Institute (CLSI, 2012) [9].

Anti-campylobacter effect of the leaf extracts of Eichhornia crassipes (*In-Vitro* Antibacterial Assay)
The agar well diffusion method was followed to determine the minimum inhibitory concentration for antimicrobial activity of the extract. The Mueller Hinton Agar plates with 5 per cent defibrinated sheep blood were swabbed with overnight culture of *C. jejuni* NCTC 11168. Wells (eight mm diameter and about two centimetres apart) were made in each of these plates using sterile cork borer. Various concentrations of the freeze dried extract were prepared in sterile distilled water for the aqueous extract. About 30 µL of different concentrations of these extracts were added in the wells and allowed to diffuse at room temperature for two hours. The plates were incubated at 42ºC for 24-48 h and zone of inhibition was measured. The minimum concentration required to inhibit the organism was also detected. The experiments were done in triplicates and the antibacterial activities were expressed as mean diameters (mm) of inhibition zones produced by the plant extract. Finally, the inhibition zones were categorized as >15 mm, 10–15 mm, and <10 mm and their activities recorded as strong, moderate, and weak, respectively.

Results and Discussion
Eichhornia crassipes, a major freshwater weed, which is a native to the Amazon basin of South America, is popular as a water ornamental around the world. This free-floating perennial herb seen in fresh water ecosystems, especially in the tropical/sub-tropical regions is enriched with plant nutrients. The antibacterial effect of the cold aqueous leaf extract of *E. crassipes* against the standard reference strain, *C. jejuni* NCTC 11168 was evaluated. The percentage yield of the extract on dry matter basis was 11.35 per cent. This Percentage Yield was slightly more than that reported by Kiristos *et al.* (2018) [10], where the ethanolic leaf extract had a percentage yield of 10.3 per cent and the methanolic leaf extract had 6.7 per cent.

Phytochemical analysis of aqueous extracts of leaf of E. crassipes
The aqueous extract of leaves of *E. crassipes* were light-brown in colour. Qualitative analysis revealed that extracts of the leaves were positive for steroids, alkaloids, phenolic compounds, flavanoids, glycosides, saponins and tritermines as is evident in table 1. The phytochemical test results and Agar well diffusion assay are as shown in fig. 2. The steroids and phenol content was more in the aqueous leaf extract.

Table 1: Phytochemical analysis of aqueous cold leaf extracts of *Eichhornia crassipes*

Sl. No.	Chemical constituent (Test)	Aqueous cold leaf extract
1	Steroids (Salkowski test)	++
2	Alkaloids (Wagner's test)	+
3	Phenolic compounds (Ferric chloride test)	++
4	Flavanoids (Lead acetate test)	+
5	Glycosides (Sodium hydroxide test)	+
6	Saponins (Foam test)	+
7	Tannins (Ferric chloride test)	-
8	Tritermines (Salkowski test)	+

(-): Indicates absence of secondary metabolite, (+): Score indicates slight positive reaction for secondary metabolites, (++): Score indicates definitive positive reaction for secondary metabolites, *E. crassipes*: *Eichhornia crassipes*

Fig 2: Phytochemical analysis and Agar well diffusion assay of the aqueous leaf extract of *E. crassipes*

Kiristos *et al.* (2018) [10] reported that the preliminary phytochemical analysis of *E. crassipes* leaves revealed the presence of major secondary metabolites like alkaloids, saponins, steroids tannins and terpenoids, which were found in both the methanolic and ethanolic leaf extracts of *E. crassipes*, while flavonoids were present only in the ethanolic extract. Haggag *et al.* (2017) [11] reported that the n-butyl leaf extracts possess secondary metabolites like flavonoids, alkaloids, terpenoids, phenol and protein. Findings of the present study were also in par with that of Isebe (2016) [12] who revealed the presence of flavonoids, terpenoids and alkaloids in the aqueous extract of *E. crassipes* in Kenya.

Anti-campylobacterial effect of E. crassipes

The antibacterial effect of aqueous cold extract of leaves of *E. crassipes* was evaluated on the standard strain *C. jejuni* NCTC 11168. The zone of inhibition of bacterial growth was evaluated by the agar well diffusion method for assessing the bacterial potential of the aqueous extracts, which is shown in table 2. Standard antibacterial (gentamicin- 10 µg/ disc) was used as positive control.

Table 2: Anti-campylobacter activity of aqueous cold leaf extract of *E. crassipes* by agar well diffusion method

Sl. No.	Type of extract	Zone of inhibition (mm)						
		Concentration of extract (mg/ mL)						
		250	200	100	50	25	12.5	6.25
1	Aqueous Cold Leaf extract	20	16	16	14	14	13	11

A linear increase in concentration of extracts resulted in an increase in the anticampylobacterial effect on the standard strain. The minimum concentration for inhibition of the organism was 25 mg/mL for the extract.

The antibacterial activity of the aqueous extract of the plant was far better than the effect produced in the findings made by Kiristos *et al.* (2018) [10], wherein the author reported that both the ethanolic and methanolic leaf extracts at 100 mg/mL, 125 mg/mL, and 150 mg/mL exhibited similar significant inhibition zone against the Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) at 12–18 mm, while a moderate zone was seen against the Gram-negative bacteria, *Escherichia coli* (*E. coli*) O157:H7 at 9–16 mm and Salmonella typhi (*S. typhi*) at 10–14 mm. The difference in antibacterial activities of the crude extracts between the bacterial strains may be attributed to the structural difference in the outer membrane, with the Gram-negative bacteria possessing different barriers for many antibiotic molecules and the crude extracts being unable to penetrate the membrane barriers. Apart from this, the periplasmic space possesses enzymes, that are capable of breaking down foreign molecules and seem to be less susceptible to plant extracts compared to Gram-positive bacteria (Shan *et al.*, 2007) [13]. Contrary to this study, Isebe (2016) [12] reported that *S. aureus* and *Bacillus subtilis* were sensitive to the crude extract, but no activity was seen against Gram negative organisms, like *E. coli* and *Salmonella typhimurium*. The present results were in concurrence with findings of Thamaraiselvi *et al.* (2012) [14], Aravind *et al.* (2013) [15], Jayanthi and Lalitha (2013) [16] and Shehnaz and Vijayalakshmi (2016) [17]. While Haggag *et al.* (2017) [11] reported n-butyl alcoholic leaf extract showed a higher inhibition zone against *E. coli* (11.3 mm) and Bacillus subtilis (23.8 mm), Kayathri *et al.* (2015) [18] reported that nbutyl alcoholic and ethanolic leaf extracts exhibited strong antibacterial activity against *S. aureus* (15 mm), followed by *E. coli* (10 mm), respectively. The present study revealed that phenolic compounds were present at a higher level in the aqueous cold leaf extract which was in par with the findings

of Klancnik *et al.* (2012) [19] who stated that phenolic compounds of plant origin has been determined to have anti-*Campylobacter* activity.

Earlier investigations have demonstrated that *C. jejuni* presents one of the greatest zoonosis hazards to public health when compared to other *Campylobacter* spp. (Gurtler *et al.*, 2005 and Platts-Mills and Kosek, 2014) [20, 21]. Decades of having to deal with emerging and ever-present foodborne bacterial culprits has resulted in devising newer and effective methods for the control and prevention of foodborne diseases without the menace of the flaring up of antibiotic resistance in pathogenic organisms. Phytoextracts can serve to counter many of the common bactrerial pathogens to some extent without causing much deleterious effects on the surroundings. Preliminary phytochemical analysis revealed that the aqueous cold leaf extract of *E. crassipes* showed broadspectrum of in-vitro antibacterial activity against the tested bacteria on account of the presence of plant derived bioactive secondary metabolites such as alkaloids, saponins, tannins, steroids and terpenoids. These bioactive compounds have antibacterial activity against bacterial infections, and thus can help to provide a protective effect. These compounds can thus serve as potential antibacterial candidates for use in the food industry, since these are plant derived and may be economical.

Conclusion

The increasing incidence of Campylobacteriosis in many parts of the world, it's duration of infection and the possible complications (1 in 100 cases), makes it a priority from a socio-economic perspective. The recognition of the public health significance of this zoonoses, it's links with the underprivileged and associated cultural traditions, along with the increasing environmental degradation activites that promote it's persistence, and the lack of tools for it's effective control is increasing. From the results of the present study, it can be concluded that the traditional use of *E. crassipes* leaves in the control of foodborne pathogens is an area of priority that could be exploited. Thus, novel and broadspectrum effective plant-derived antibacterial agents, could be utilised for the control of food pathogens.

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Competing Interests

The authors declare that they have no competing interests.

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