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Research Article

TURBIDIMETRIC ASSAY REITERATES THE BACTERIOSTATIC ACTION OF *CENTELLA ASIATICA* AGAINST *E.COLI, B. SUBTILLIS* AND *S. MUTANS*

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ABSTRACT
Centella asiatica is a nootropic herb growing in Asian subcontinent including India. Besides its mind boosting and neuroprotective action, it is used for the treatment of wide range of ailments such as skin diseases, urinary tract infections, wound healing etc. This might be due to its antimicrobial activity. Therefore we have earlier studied the effect of fresh leaf juice of *Centella asiatica* against *E.coli, B. subtillis* and *S. mutans* by disc diffusion and agar well diffusion method and we found that it exerts bacteriostatic action. In the present investigations the effect of fresh leaf juice of *Centella asiatica* was studied by turbidimetric assay which also demonstrated the bacteriostatic action against these bacteria. Thus fresh leaf juice of *C. asiatica* was found to possess antibacterial activity against *B. subtilis, S. mutans* and *E.coli*. The MIC of leaf juice was higher for *E.coli* (MIC 2.5%) than *B. subtilis* (MIC 0.156%), *S. mutans* (MIC 1.25%). However, there was enhanced growth of *E. coli* at lower concentration of leaf juice. Such enhanced growth was not observed in *B. subtilis* and *S. mutans*.

Keywords: *Turbidimetric assay, Antibacterial activity, C.asiatica, E.coli, B. subtilis, S.mutans, MIC*

INTRODUCTION
Centella asiatica (Hydrocotyle asiatica) is also known as Gotu kola, Indian pennyworts and Mandukparni. It belongs to family Umbelifereae. It is found in swampy areas, as a weed in crop fields and other waste places throughout the India. It has been used for various ailments viz. for boosting memory, increasing concentration and alertness, anti-anxiety, anti-stress and as a mild diuretic (Ganachari *et al*. 2004). *Centella asiatica* has been used as a constituent of a brain tonic for mentally retarded children (Inamdar *et al*. 1996). Methanolic and elthyl acetate extracts of *Centella asiatica* and pure asiaticoside, imparted anxiolytic activity (Wijeweera *et al*. 2006). Besides the neurological disorders, it is also used for wound healing, for the treatment of various skin disorders like leprosy, lupus, varicose ulcers, eczema, psoriasis and also recommended in diarrhoea, fever, amenorrhea and diseases of the female genitourinary tract (Gohil *et al*. 2010). The wound healing property of *Centella asiatica* may be due to its antimicrobial effect. Earlier we have demonstrated the antibacterial activity of fresh leaf juice of *Centella asiatica* on *E.coli, B. subtillis* and *S. mutans* (Chaudhari et al. 2013) by disc diffusion and agar well diffusion method. In the present investigations the effect of fresh leaf juice of *Centella asiatica* was studied by turbidimetric assay.

MATERIAL AND METHODS
The saplings of *Centella asiatica* were obtained from Aamzari (Melghat Tal. Chikhaldara, Maharashtra, India) and planted in the departmental garden. The plant species was authenticated at Department of Botany, Govt. Vidarbha Institute of Science and Humanities, Amravati. The Fresh leaf juice of *Centella asiatica* was prepared as per the protocol described earlier (Chaudhari et al 2013).

Test microorganisms:
Escherichia coli (MTCC 739), *Bacillus subtilis* (MTCC 736), *Streptococcus mutans* (MTCC 890) were procured from the Institute of Microbial Technology (IMTECH), Chandigarh. *Escherichia coli* (MTCC

739), *Bacillus subtilis* (MTCC 736) were suspended in nutrient broth and incubated at 37°C for 24 hrs. *Streptococcus mutans* (MTCC 890) was suspended in brain heart infusion broth and incubated at 37°C for 48 hrs. Before 24 hrs of experimentation, a loopful suspension from the broth was inoculated on slant of respective media and incubated at 37°C to obtain the culture in logarithmic phase.

Study groups:

Experimental group: In this group, test organisms were grown in nutrient broth/brain heart infusion broth containing serially diluted fresh leaf juice.

Ciprofloxacin group: Ciprofloxacin was used as the positive control.

Control for colour of leaf juice: Since the leaf juice is green in colour which changes with time. Therefore, a set of 12 test tubes containing serially diluted leaf juice without inoculum was prepared and incubated along with the experimental tubes.

Determination of Minimum Inhibitory Concentration (MIC):

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. The MICs were determined by the broth microdilution method recommended by the NCCLS (1997). For determining MIC, the leaf juice was serially diluted in nutrient broth/brain heart infusion broth. Each test tube was inoculated with a loopful suspension of the test organism. The highest concentration of leaf juice in the first test tube was 10µl/ml, and then the concentration was reduced to half in each successive tube by serial dilution. Blank tube contained only culture medium, whereas, the control tube contained culture medium inoculated with a loopful suspension of the test organism. All the tubes were incubated at 37°C for 24 hours.

After completion of the incubation period, all the tubes were transferred to ice bath to retard the growth of bacteria and observed for MIC. The lowest concentration of the leaf juice or the antibiotic where no visible growth was observed was considered as the minimum inhibitory concentration.

In the ciprofloxacin treated group, MIC of ciprofloxacin for test organisms was determined by similar method. The concentration of ciprofloxacin was 50µg/ml in the first tube.

Turbidimetric assay (*Li et al. 1993)****:*** After determining the MIC, O.D. of each sample was read spectrophotometrically at 600nm to quantify the bacterial growth.Sterile nutrient broth/ brain heart infusion broth inoculated with a loopful suspension of the test organism was used as a blank.

RESULTS

The results of MICs of leaf juice of *Centella asiatica* and ciprofloxacin are displayed in table No. 1 and 2 respectively. The MIC of leaf juice of *Centella asiatica* for *E.coli* was 2.5%, for *B.subtilis*, 0.156% and for *S. mutans* 1.25%. In turbidimetric assay, *E. coli* exhibited no growth up to the concentration 2.5% which is MIC. Below this concentration, there was increased growth of *E. coli*. The optical densities of these tubes were higher than the blank. This indicates that at lower concentration, leaf juice of *C. asiatica* did not inhibit the growth of *E. coli* but enhanced the growth. For *B. subtilis,* in turbidimetric assay, leaf juice of *Centella asiatica* demonstrated inhibitory effect up to 0.16%. Below this concentration, there was growth of *B. subtilis,* which was four times lower than the control. For *S. mutans* the leaf juice was showing inhibitory effect up to the concentration of 1.25%. MIC of ciprofloxacin was 0.078 µg/ml for *E. coli*, 0.039 µg/ml for *B. subtilis* and 1.25µg/ml for *S. mutans*.

DISCUSSION

The MIC of leaf juice for all the three test organisms was far higher than the MIC of the Ciprofloxacin. This could be due to the crude nature of the leaf juice, which predominantnly contained water. The active principles which show antibacterial properties are very low in quantity in the leaf juice. According to Ovedeji and Afolayan (2005) *Centella asiatica* contains Centella Saponins B, C and D. Mandal *et. al.* (2005) found that Saponins isolated from *Acasia auriculiformi*s possess antibacterial and antifungal activity. Saponins from *Gymnema sylvestre* and *Eclipta prostrata* exhibited antibacterial and antifungal activity (Khanna and Kannabiran 2008). According to Segal *et al.* (1974) saponins interact with the

Table 1: Minimum Inhibitory Concentration of Fresh leaf Juice of *Centella asiatica* for the test organisms

Microorganism	Concentration of Fresh Leaf Juice of *Centella asiatica* in %											Blank	Result
	20	10	05	2.5	1.25	0.63	0.31	0.156	0.078	0.039	0.0195	0.00	MIC in %
E. coli	-	-	-	-	+	+	+	+	+	+	+	+	2.5
B.subtilis	-	-	-	-	-	-	-	-	+	+	+	+	0.156
S. mutans	-	-	-	-	-	+	+	+	+	+	+	+	1.25

- sign indicates no growth + sign denotes appearance of visible growth

Table 2: Minimum Inhibitory Concentration of Ciprofloxacin for the test organisms.

Microorganism	Concentration of Ciprofloxacin (µg/ml)											Blank	Results
	10	05	2.5	1.25	0.625	0.31	0.156	0.078	0.039	0.0195	0.00976	0.00 µg/ml	MIC in µg/ml
E. coli	-	-	-	-	-	-	-	-	+	+	+	+	0.078
B. subtilis	-	-	-	-	-	-	-	-	-	+	+	+	0.039
S. mutans	-	-	-	-	+	+	+	+	+	+	+	+	1.25

- sign indicates no growth + sign denotes appearance of visible growth

Table 3: Optical densities showing growth of the test orgamisms in turbidimetric assay

Test tube No.	Concentration of Fresh Leaf juice	O.D. due to the growth of the test organism		
		E.coli	***B.subtilis***	***S. mutans***
1	20%	0.01	0.04	0.06
2	10%	0.02	0.03	0.07
3	5%	0.03	0.03	0.06
4	2.50%	0.02	0.04	0.07
5	1.25%	0.17	0.04	0.08
6	0.63%	0.13	0.03	0.17
7	0.31%	0.17	0.02	0.16
8	0.156%	0.12	0.02	0.17
9	0.78%	0.15	0.18	0.26
10	0.39%	0.16	0.18	0.25
11	0.20%	0.13	0.19	0.25
Control	0%	0.1	0.8	0.31

membrane lipids causing generation of pores in the plasma membrane. Avato *et al.* (2006), studied the antibacterial activity of saponins from Medicago species and found that these saponins are more active against Gram positive bacteria. In the present investigations, the MICs of fresh leaf juice of *C. asiatica* for Gram positive bacteria *B. subtilis* and *S. mutans* were 0.156% and 1.25%, whereas, for *E. coli* which is a Gram negative bacterium, it was 2.5% indicating that saponins are more active in Gram positive bacteria. According to Arabaski *et al.* (2012) saponins may distub the permeability of the bacterial lipopolysachharide layer in Gram negative bacteria. This might be the reason of antibacterial effect of fresh leaf juice of *C. asiatica* against *E. coli*. Deshmukh *et al.* (2007) found the antibacterial activity of fresh leaf juice of *Tridax procumbens* on Gram positive bacteria *S. aureus and B. subtilis* but it was no any effect on *E.coli.* The results of our earlier studies by disc diffusion method and agar well diffusion method also exhibited similar antibacterial activity of the fresh leaf juice of *C. asiatica* (Chaudhari *et al.* 2013).

According to Inamdar *et al.* (1996), *Centella asiatica* also contains pentacyclic triterpenes such as asiatic acid, asiaticoside, madecassic acid, madecasoside as active priciples. Haraguchi *et. al.* (1999) found that triterpenes like rotundic acid, ursolic acid and peduncloside isolated from *Ilex integra* showed significant broad spectrum antimicrobial activity against bacteria, yeast and filamentous fungi. According to Kalita and Saikia (2012), triterpenes in *C. asiatica* bind to the bacterial membrane causing its disruption. This might be the reason of antibacterial activity observed in the present study. Thus, the present investigations of the turbidimetric study reiterates the antibacterial action of fresh leaf juice of *Centella asiatica.*

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