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Research Article

EVALUATION OF ANTIBACTERIAL PROPERTY OF *CENTELLA ASIATICA*

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ABSTRACT

Centella asiatica is a medicinal herb, known as mandukparni or Gotu-kola. It is used in Ayurvedic, Chinese, Unani, and other folk systems of medicines. In the present investigations, the fresh leaf juice of *Centella asiatica* was used for the evaluation of antibacterial property. For this study, *Escherichia coli* (MTCC 739), *Bacillus subtilis* (MTCC 736), *Streptococcus mutans* (MTCC 890) and *Staphylococcus aureus* (ATCC 6538) were used. Fresh leaf juice of *C. asiatica* produced a zone of inhibition in disc diffusion and agar well diffusion method for *E. coli*, *B. subtilis* and *S. mutans* after 24 hrs of incubation at 37^{o}C. In the plate of *E.coli* the zone of inhibition was found to be followed by a narrow zone of enhanced growth. This showed that lower concentration of leaf juice stimulates growth of *E.coli*. When the incubation was further continued for another 24 hrs, growth of microorganisms was observed in the zone of inhibition indicating that the effect was bacteriostatic for *E.coli*. Thus, fresh leaf juice of *C. asiatica* was found to possess antibacterial activity against *B. subtilis, S. mutans* and *E.coli*.

Key words: *C.asiatica, E.coli, B. subtilis, S.mutans, Antibacterial Activity*

INTRODUCTION

In a constant attempt to improve the quality of life, human used plants as a source of food, shelter, clothing, medicine and cosmetics. Many plants possess medicinal properties due to having secondary metabolites which help to cure various ailments (Sliva Junior *et al.* 1994). Nature has provided a wide range of medicinal plants that can provide a remedy against various diseases as well as disorders. From ancient time, the use of medicinal plants and their applications for mankind had been in practice. Most of these medicines are found to be effective without adverse side effects since time immemorial. Still, these medicines are not routinely used due to lack of well-documented trials. Knowledge of medicinal plants is limited to certain communities and ethnic groups (Di Stasi 1996). So there is need to understand and unravel the medicinal properties of plants and plant products. In the present investigations antibacterial activity of *Centella asiatica* was studied against *E.coli, B. subtilis, S. mutans* and methicillin resistant *Staphylococcus aureus.*

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 of India, commonly found as a weed in crop fields and other waste places throughout India. It is used as memory enhancer cognitive enhancer, anti-anxiety, anti-stress, wound healing drug and as a mild diuretic. *Centella asiatica* has been used as a constituent of brain tonic for mentally retarded children (Inamdar *et al*. 1996). It has also been used traditionally and in Ayurvedic medicine for CNS ailments, including memory loss, insomnia, depression, stress, epilepsy, anxiety and for improving cognition (Ganachari *et al*. 2004). Methanolic and ethyl 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). In the present investigation antibacterial activity was studied against *Escherichia coli, Bacillus subtilis, Streptococcus mutans* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Collection and cultivation of Centella asiatica:

The *Centella asiatica* was collected from Aamzari (Melghat Tal. Chikhaldara, Maharashtra, India) and was authenticated at Department of Botany, Govt. Vidarbha Institute of Science and Humanities, Amravati. It was then cultivated in the departmental garden.

Preparation of leaf juice of Centella asiatica:

Fresh leaf juice was prepared by crushing the washed leaves and filtering it through sixteen layered muslin cloth.

Preparation of decoction of Centella asiatica:

Powder of shed dried leaves of *Centella asiatica* was soaked in warm distilled water and maintained on low flame for 45 minutes until it was reduced to half of the original volume. The decoction was allowed to cool and filtered through 16 layered sterile muslin cloth.

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^{o}C for 24 hrs. *Streptococcus mutans* (MTCC 890) was suspended in brain heart infusion broth and incubated at 37^{o}C for 48 hrs. *Staphylococcus aureus* (ATCC 6538), which is a methicillin resistant strain was also used in this study.

Before 24 hrs of experimentation, a loopful suspension from the broth was inoculated on slant of respective media and incubated at 37^{o}C to obtain the culture in logarithmic phase.

Preparation of culture media:

I. Nutrient broth and Nutrient agar:

i. Nutrient broth:

Nutrient broth was prepared by dissolving peptone (Peptic digest of animal tissue) (HiMedia) 5.0 gm, Sodium chloride (Qualigen) 5.0 gm, Beef extract (HiMedia) 1.5gm, Yeast extract (HiMedia) 1.5gm in 1000 ml of glass distilled water. The solution was heated to dissolve the ingredients completely. Final pH was adjusted to 7.4±0.2 and sterilized by autoclaving at 15 lbs /sq. Inch (121°C) for 15 minutes.

ii. Nutrient agar:

The nutrient agar was prepared in a similar way and 1.5g Agar agar (HiMedia) was added along with other constituents.

II. Brain heart infusion Broth and Brain heart infusion agar:

i. Brain heart infusion broth:

3.7g of Brain Heart infusion Broth ready to mix powder (Hi-media) was dissolved in 100ml of distilled water by heating and pH was adjusted to 7.4 ± 0.2 and autoclaved at 15lbs/sq. inch (at 121°C) for 15 min.

ii. Brain heart infusion agar: To prepare brain heart infusion agar 5.2 g of Brain Heart Infusion Agar powder (HiMedia) was dissolved in 100 ml of distilled water by heating and pH was adjusted to 7.4 ± 0.2 and autoclaved at 15lbs/sq. inch (at 121°C) for 15 min.

Preparation of Slants and plates: Nutrient agar and brain heart infusion agar was used to prepare slants and plates. Slants were prepared in the test tubes while 90 mm petri dishes were used for preparation of plates. *E.coli*, *S. aureus* and *B. subtilis* were grown on nutrient agar slants while *S. mutans* and oral bacterial isolates were grown on Brain heart infusion agar slants.

Preparation of inoculum: Optical Density of an inoculum is directly proportional to the number of cells present in it. The inoculum was prepared by suspending the bacterial colonies obtained from overnight-incubated culture (in logarithmic phase) into prechilled 0.9 % saline and the OD was set to 0.2.

Antibacterial study: For this purpose, fresh leaf juice and decoction of dried leaves were used. Ciprofloxacin a broad-spectrum bactericidal antibiotic was used as a positive control.

i. Disc diffusion method **(**Baurer *et al.* 1966**)**:

Sterilized Whatman filter paper No.1 discs of the diameter 5mm were soaked in 5µl of fresh leaf juice/decoction of dried leaf powder. The discs were placed on the surface of solid agar plates on

which, 0.1ml of the inoculum of test organism was spread uniformly. The plates were initially transferred to refrigerator for 30 min. to allow the diffusion of the contents from the disc to the surface of agar and then incubated at 37°C for 24 hrs. The diameter of the zone of inhibition was measured and plates were incubated for another 24 hrs.

ii. Agar well diffusion method **(**Perez *et al.* 1990**):**

0.1ml of the inoculum of test organism was spread using sterile glass spreader on the surface of nutrient agar. After inoculation , the inoculum was allowed to set for 30 min at 4°C. Wells of the diameter of 3 mm were then punched in the agar using sterile gel puncher and filled with 10 µl of the sample. The plates were kept at 4°C for 30 min to allow diffusion of the content from the well. Then the plates were incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured and plates were incubated for another 24 hrs.

Statistical analysis:

Each experiment was carried out for six times. Arithmetic mean and standard deviation were calculated using six observations of these trials. The Significance of the results was determined by unpaired Student t test.

RESULTS

a. Disc diffusion method:

The results of the disc diffusion method are shown in table No1. Fresh leaf juice of *Centella asiatica* exhibited a zone of inhibition of 16.00 ± 5.6mm in diameter for *E. coli* (Plate 1- Fig 1). A narrow rim of the zone of enhanced growth was seen immediately after the zone of inhibition. In ciprofloxacin group the zone of inhibition was 38.75 ± 3.06mm in diameter. Decoction of dried leaves did not show any inhibition for *E. coli* (Plate1- Fig 3).

Fresh leaf juice of *Centella asiatica* exhibited a zone of inhibition of 16.57 ± 1.72mm in diameter for *B. subtilis* and 15.25 ± 1.26mm in diameter for *S. mutans* (Plate 2- Fig 1 and Plate 3- Fig 1). Control group (ciprofloxacin) showed the zone of inhibition of 45.29 ± 0.76 mm in diameter for *B. subtilis* and 38.75 ± 1.89 mm in diameter for *S. mutans*. Decoction of dried leaves did not show any inhibitory action against these test organisms (Plate 2- Fig 3 and Plate 3- Fig 3).

Neither the fresh leaf juice nor the decoction of dried leaves of *Centella asiatica* exhibited any effect on *S. aureus*. Control group showed 34.86 ± 1.07mm zone of inhibition for *S. aureus* surrounding the disc soaked in ciprofloxacin (Plate 4- Fig 1 and Fig 3).

Table 1: Effect of *Centella asiatica* on test microorganisms studied by Disc diffusion Method showing diameter of zone of inhibition in mm.

Microorganism	Effect of fresh Leaf Juice		Effect of decoction	
	Control group	Experimental group	Control group	Experimental group
E.Coli	38.75 ± 3.06	16.00 ± 5.60	38.75 ± 3.06	00.00
B.subtilis	45.29 ± 0.76	16.57 ± 1.72	45.29 ± 0.76	00.00
S. mutans	38.75 ± 1.89	15.25 ± 1.26	38.75 ± 1.89	00.00
S. aureus	34.86 ± 1.07	00.00	34.86 ± 1.07	00.00

b. Agar well diffusion method:

The results of Agar well diffusion method are shown in table No.2. Fresh leaf juice of *Centella asiatica* inhibited the growth of *E. coli* and the zone of inhibition was 13.14 ±1.6mm in diameter (Plate 1-Fig 2). The zone of inhibition was followed by a narrow zone of enhanced growth. For ciprofloxacin, it was 40 ± 0.58mm in diameter. The decoction of dried leaf powder of *Centella asiatica* was ineffective for *E. coli* (Plate 1-Fig 4).

A zone of inhibition of 13.86 ± 2.19 mm in diameter was observed for *B. subtilis* and 14.00 ± 0.816 mm in diameter for *S. mutans* surrounding the well containing fresh leaf juice of *Centella asiatica*

(Plate 2- Fig 2 & 3- Fig 2). Zone of inhibition of 42.29 ± 3.45mm and 37.75 ± 1.96mm were observed for *B. subtilis* and *S. mutans* respectively around the well containing ciprofloxacin.

Plate 1 Effect of *C. asiatica* on *E. Coli* (C: Ciprofloxacin, P: Plant product)

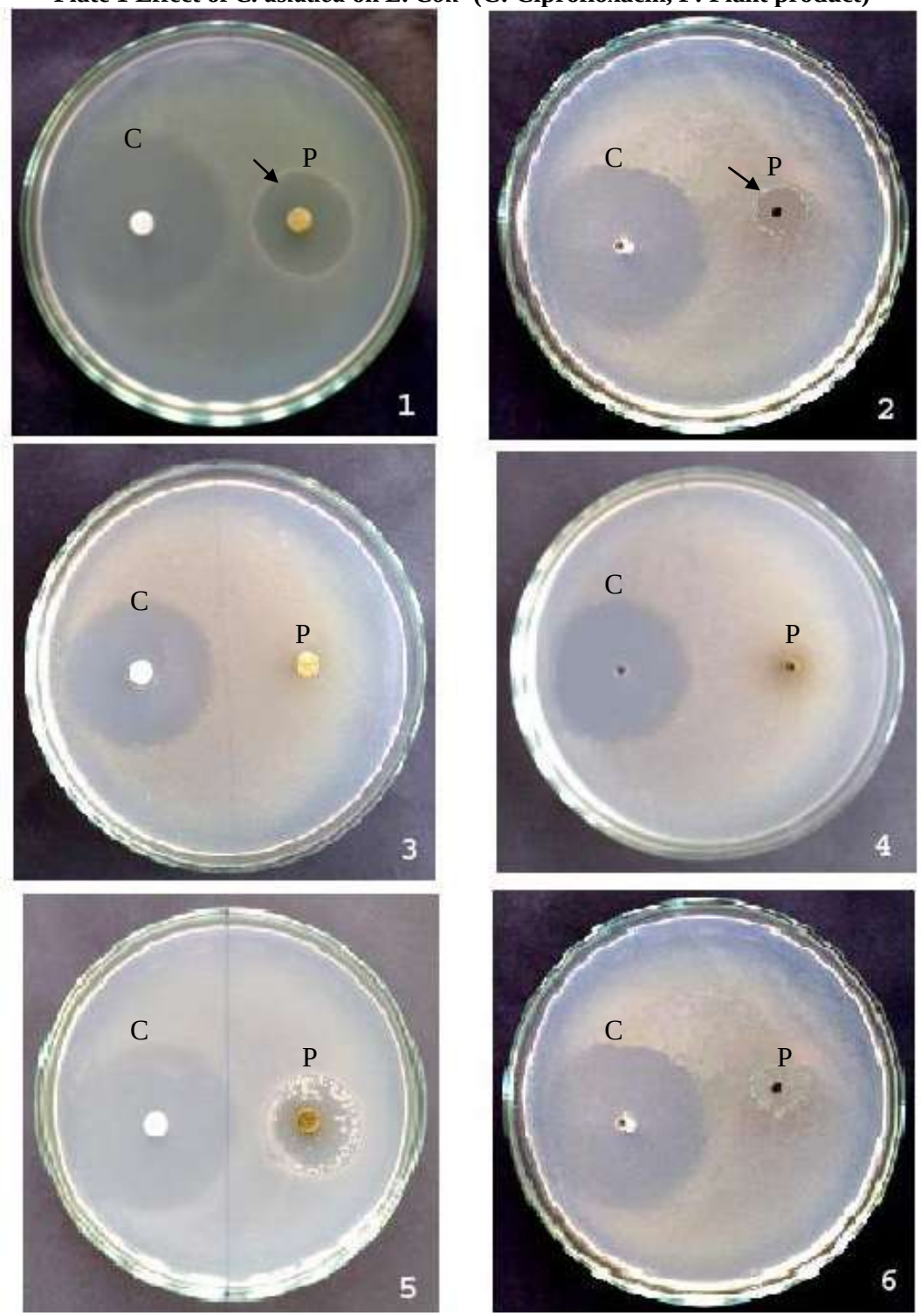

Fig 1, 3, 5: Results of Disc diffusion method and Fig 2,4,6: Results of agar well diffusion method

Fig 1,2: Effect of Leaf Juice showing zone of inhibition followed by zone of enhanced growth. (Arrow)

Fig 3,4: Effect of decoction. No zone of inhibition

Fig: 5,6: Growth of *E. Coli* after 48 hrs of incubation, showing bacteriostatic effect.

Plate 2 Effect of *C. asiatica* on *B. subtilis* (C: Ciprofloxacin, P: Plant product)

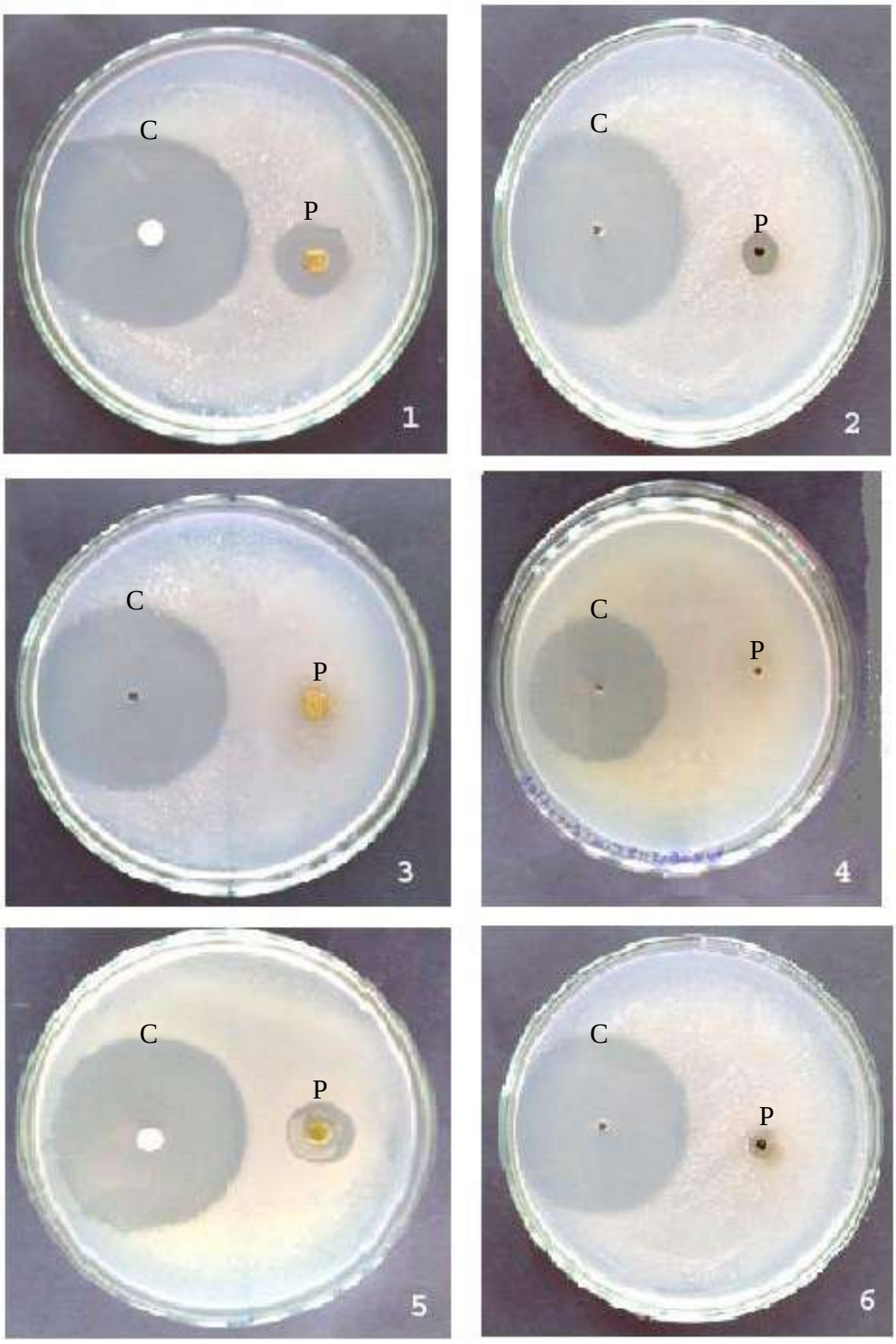

Fig 1, 3, 5: Results of Disc diffusion method **Fig 2,4,6: Results of agar well diffusion method**

Fig 1,2: Effect of Leaf Juice showing zone of inhibition

Fig 3,4: Effect of decoction. No zone of inhibition

Fig: 5,6: Growth of E. Coli after 48 hrs of incubation, showing bacteriostatic effect.

Plate 3 Effect of *C. asiatica* on *S. mutans* (C: Ciprofloxacin, P: Plant product)

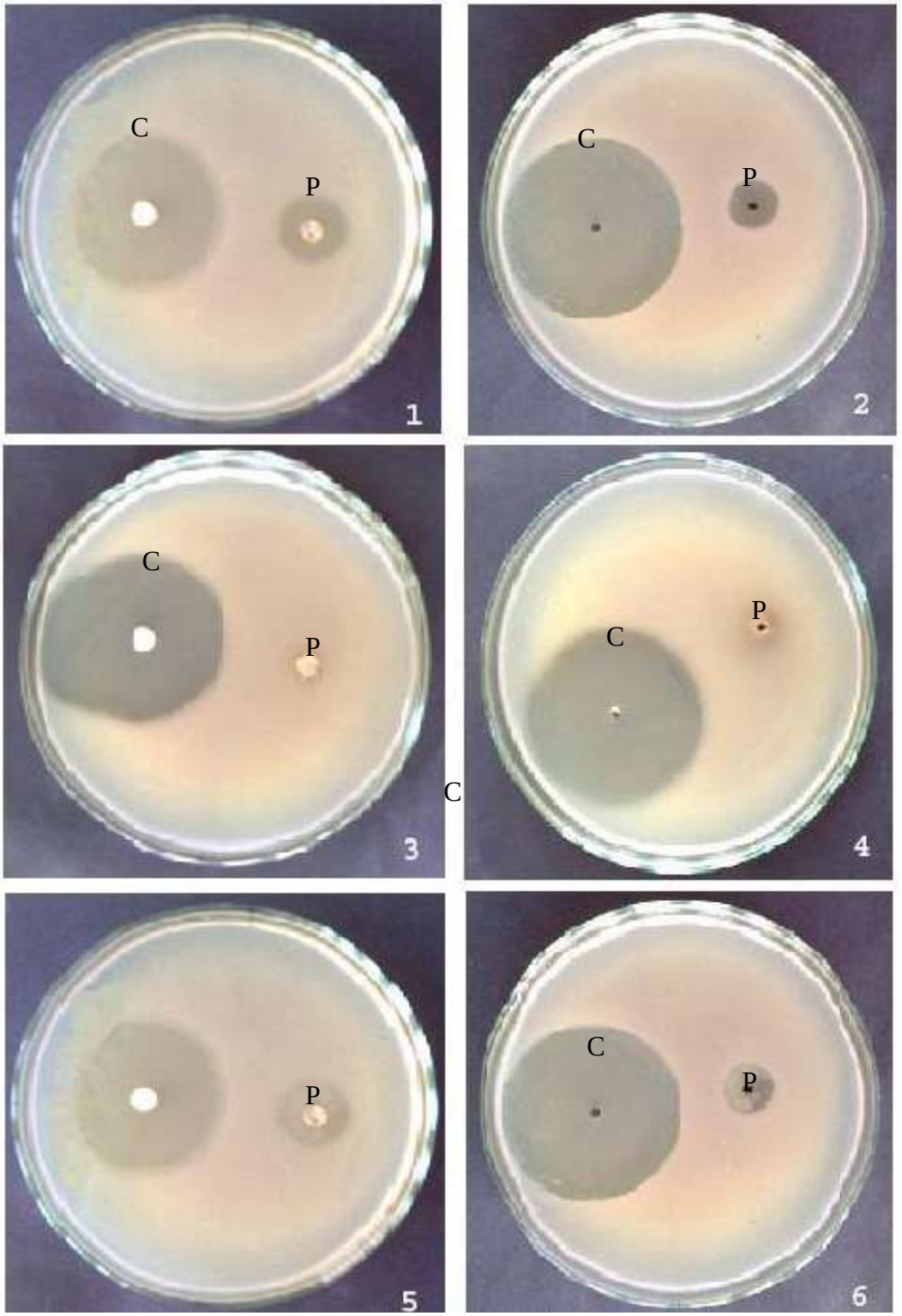

Fig 1, 3, 5: Results of Disc diffusion method **Fig 2,4,6: Results of agar well diffusion method**

Fig 1,2: Effect of Leaf Juice showing zone of inhibition

Fig 3,4: Effect of decoction. No zone of inhibition

Fig: 5,6: Growth of E. Coli after 48 hrs of incubation, showing bacteriostatic effect.

C

P

Plate 4 Effect of *C.asiatica* on *S. aureus* (C: Ciprofloxacin, P: Plant product)

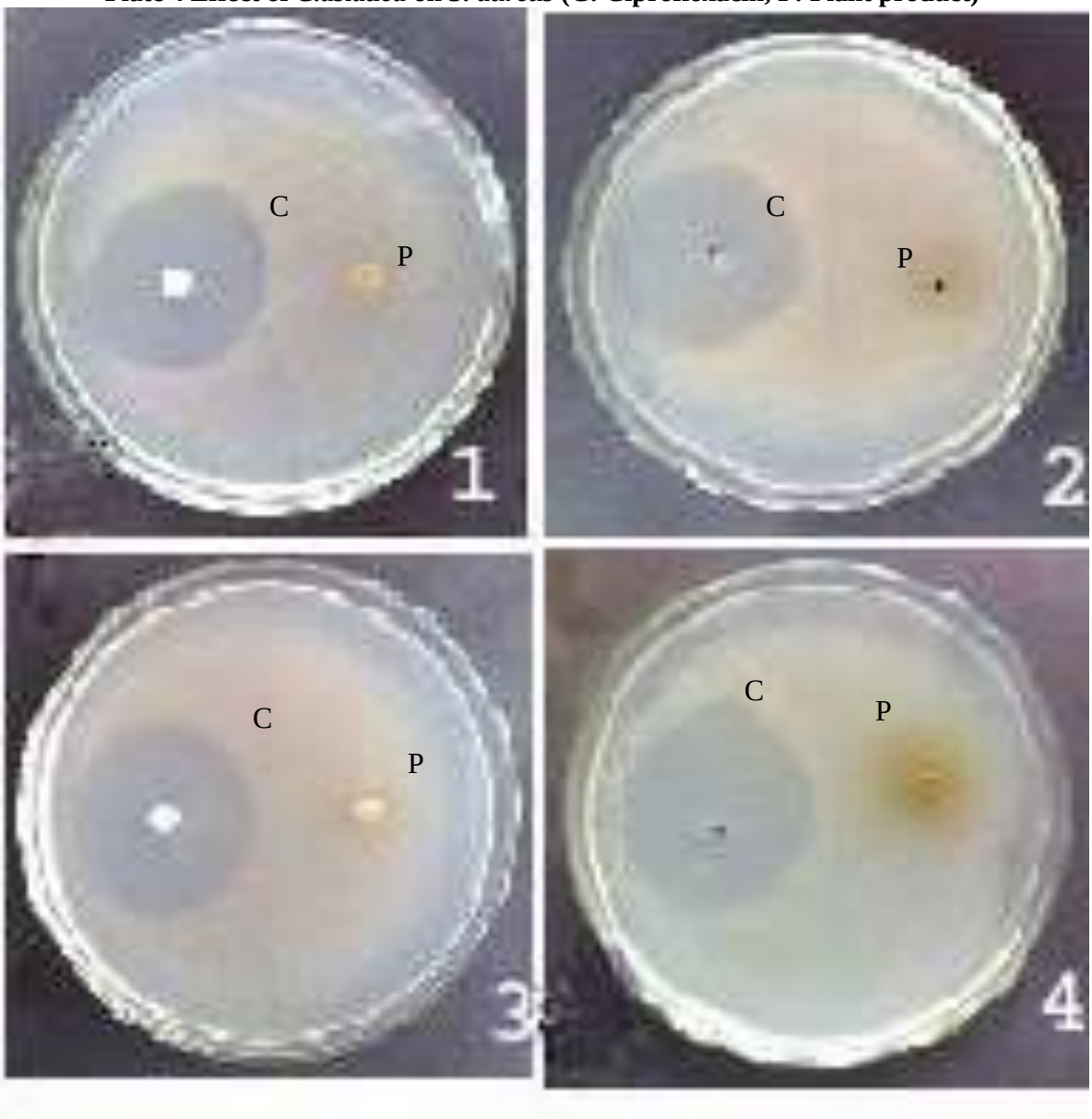

Fig 1, 3,: Results of Disc diffusion method **Fig 2,4,: Results of agar well diffusion method**
Fig 1,2: Effect of Leaf Juice showing no zone of inhibition
Fig 3,4: Effect of decoction. No zone of inhibition

Decoction of dried leaf powder of *Centella asiatica* did not show any effect on *B. subtilis* and *S. mutans* (Plate 2- Fig 4 and Plate 3- Fig 4).

For *S. aureus* there was a zone of inhibition of 36.2 ±1.6 mm surrounding the well containing ciprofloxacin, but fresh leaf juice and decoction of dried leaves powder of *Centella asiatica* did not show any inhibitory activity (Plate 4- Fig 2 and Fig 4).

Table 2: Effect of *Centella asiatica* on test microorganisms studied by Agar well diffusion method showing diameter of zone of inhibition in mm.

Microorganism	Effect of fresh Leaf Juice		Effect of decoction of dried leaves	
	Control group	Experimental group	Control group	Experimental group
E.coli	40.00 ± 0.58	13.14 ± 1.68	40.00 ± 0.58	00.00
B.subtilis	42.29 ± 3.45	13.86 ± 2.19	42.29 ± 3.45	00.00
S. mutans	37.75 ± 1.96	14.00 ± 0.816	37.75 ± 1.96	00.00
S. aureus	36.29 ± 1.60	00.00	36.29 ± 1.60	00.00

DISCUSSION

Active principles of *Centella asiatica* are pentacyclic triterpenes namely asiatic acid, asiaticoside, madecassic acid, madecassoside (Jacinda and Dubery 2009), Centella saponines B, C and D (Oyedeji and Afolayan 2005). The observed antimicrobial activity of fresh leaf juice of *Centella asiatica* against *E. coli*, *B. subtilis* and *S. mutans* may be due to the action of these active principles.

Saponins are divided into two groups, steroidal saponins and triterpenoid saponins. Saponins are glycosides occurring widely in plants. Most of the Saponins are found to possess antibacterial property. Saponins isolated from *Medicaso sp.* were found to be active especially against Gram positive bacteria. (Avato *et. al.* 2006). Khanna and Kannabiran (2008) have found that saponin fractions of *Gymnema sylvestre* and *Eclipta prostrata*, possess significant antibacterial and antifungal activity. Acaciaside A and B isolated from *Acacia auriculiformis* are bisglycoside saponins that show significant antibacterial and antifungal activity (Mandal *et. al.* 2005). Similarly, the antibacterial activity of *Centella asiatica* against *E. coli*, *B. subtilis* & *S. mutans* may be due to presence of saponins in it.

Triterpenes extracted from various plants were found to possess antimicrobial properties. Ether extract from the bark of *Poulsenia armata* contains triterpene derivatives, showed antimicrobial activity (El-Seedu 2005). Triterpenes like rotundic acid, ursolic acid and peduncloside isolated from *Ilex integra* showed significant broad spectrum antimicrobial activity against bacteria, yeast and filamentous fungi (Haraguchi *et. al.* 1999). Leaf extracts of *Syzygium guineense*, which contains ten titerpenes including asiatic acid. The asiatic acid fraction showed the most significant antibacterial activity against *E. coli* and *B. subtilis* (Djoukeng *et. al.*2005). One of the components in *Centella asiatica* is asiatic acid. This might have inhibited *E. coli* and *B. subtilis* in the region surrounding the well or disc where it diffused.

Methanolic root extract of *Aceriphyllum Rossii* was found to be potent inhibitor of growth of various strains of *S. aureus* including methicillin resistant *S. aureus*. Its active compound is Olean-27-carboxylic acid. (Zheng *et. al.*2008). Leaf juice of *C. asiatica* did not show inhibition to *S. aureus*. The components presents in leaf juice of *Centella asiatica* were not found to be active against methicillin resistant *S. aureus.* The triterpenes present in *Centella asiatica* may not have any effect on *S. aureus*. Concentration of the component effective against *S. aureus* might be very low in crude leaf juice of *Centella asiatica*, which might be also the reason for not getting inhibitory effect.

Triterpenes namely trichomycins A and B isolated from *Tricholoma sp.* are antibacterial in nature (Ovenden *et. al.* 2005). Similarly pentacyclic triterpenes isolated from *Myricaria elegansi* exhibited significant antibacterial activity (Manzoor *et. al.* 2008). *Centella asiatica* also have pentacyclic triterpenes like asiatic acid, asiaticosides etc. These triterpenes also have antibacterial property and therefore inhibited growth of *E. coli*, *B. subtilis* and *S. mutans*. Activity of Triterpenes is due to a change of membrane permeability arising from membrane lipid alterations (Haraguchi *et al*. 1999). In the present investigation also the antimicrobial activity of *C. asiatica* might be due to similar action of triterpenes, which are active constituents of *C. asiatica.*

Another component, which also possesses antimicrobial activity, is monoterpene. Essential oils from *Mentha pulegium* L. contain thirty-four oxygen monoterpene hydrocarbons displayed antimicrobial activities (Rieks *et. al.* 2004). The essential oil from *C. asiatica* also contains 11 monoterpenoid

hydrocarbons and 9 oxygenated monoterpenoids (Oyedeji and Afolayan 2005), which may contribute antimicrobial activity of *C. asiatica*.

Sesquiterpenoids are also important components of *C. asiatica*, which have antibacterial activity. There are 14 sesquiterpenoid hydrocarbons, 5 oxygenated sesquiterpenoid and 1 sulphide sesquiterpenoid found in *C. asiatica* (Oyedeji and Afolayan 2005). Sesquiterpenoids isolated from *Centipeda minima* were found to be active against eight different microbial pathogens (Liang *et al*. 2007). Sesquiterpenes isolated from *Varthemia iphionoides* exhibited potent antimicrobial activity against six bacterial species including *S. aureus, B. subtilis, E. coli* (Al Dabbas *et al*. 2005). Sesquiterpenoids isolated from roots and stems of *Ferula kuhistanica* were found to be toxic against Gram-positive bacteria (Tamemoto *et al*. 2001). Similarly sesquiterpenoid - hongoquercins A exhibited moderate activity against Gram-positive bacteria (Roll *et al*. 1998). Seven sesquiterpenoids compound isolated from *Commiphora mukul* showed wide range of inhibiting activity against both Gram positive and Gram negative bacteria (Saeed and Sabir, 2004). Rabe and Staden (2000) have isolated a sesquiterpenoid from *Warhurgia salutaris* which exhibited antimicrobial activity against Gram positive bacteria. Sesquiterpenoids accumulated in cotton leaves and cotyledons can form aqueous solution capable of inhibiting the bacterial pathogen (Essenberg *et al*. 1990). In our investigation also we have found that leaf juice of *C. asiatica* was active against *E. coli*, *B. subtilis* and *S. mutans*. This activity may be at least partially contributed by the sesquiterpenoid components of *C. asiatica*.

The zone of inhibition surrounding the leaf juice containing well or disc remain up to 24 hrs of incubation. After that there was growth of respective microorganisms in zone of inhibition after incubation for another 24 hrs. This may be due to inactivation of components of leaf juice of *C. asiatica*. So the results indicate bacteriostatic action of leaf juice rather than bactericidal action. In *E.coli*, there was a zone of enhanced growth at the margin of zone of inhibition, suggesting that at the lower concentration the fresh leaf juice of *Centella asiatica* has growth stimulatory property. Such results were also observed for the fresh leaf juice of *Tridax procumbens* earlier in our laboratory (Deshmukh et al. 2007)

Gnanamani *et al*. (1983) have shown that the antibacterial effect may be due to the pore formation in the cell wall and the leakage in cytoplasmic constituents by the active compounds present in *Acanthus montanus*. The active compounds of this plant include saponins and triterpenes, which are also the components of *C. asiatica*. Therefore, the observed antibacterial activity of leaf juice of *C. asiatica* might be due to damage to the cell wall. Mamtha *et al*. (2004) have shown antibacterial activity of *C. asiatica* against enteric pathogens.

From the results of leaf juice and ciprofloxacin, it appears that ciprofloxacin is superior over *C. asiatica* leaf juice. But the leaf juice was crude and contains large amount of water and other constituents. The active principles, which possess antibacterial properties, are in very low quantity in the leaf juice. Secondly rate of diffusion of leaf juice through agar and disc may be low as compared to ciprofloxacin. Therefore the activities of leaf juice and ciprofloxacin are not comparable.

Thus fresh leaf juice of *C. asiatica* has antibacterial activity. It was found to possess antibacterial activity against Gram positive *B. subtilis* and *S. mutans* as well as against Gram negative *E. coli*. It was not effective on methicillin resistant *S. aureus.*

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