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PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITY OF *CEROPEGIA JUNCEA* ROXB. – A RARE MEDICINAL PLANT

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ABSTRACT

The phytochemical compounds and antimicrobial activity of different solvent extracts of *Ceropegia juncea* Roxb. were screened. The qualitative analysis of crude extracts revealed the presence of alkaloids in all solvent extracts. There was a resemblance in occurrence of saponin and steroids in methanol and aqueous extracts, but phenolic compounds and tannins were found common in the extracts prepared with ethyl acetate, chloroform and water. The crude extracts exhibited MIC values ranging between 20 and 80 mg/ml. However, it was observed that extracts at 40-60 mg/ml were found to be MIC for most of the test microorganisms in this study. The antimicrobial observations exemplified significant inhibitory effect of methanolic extract against *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* and aqueous extract for *Klebsiella pneumoniae* and *Proteus vulgaris* as compared to positive control amikacin. The results provide baseline information for the use of the solvent extracts of *Ceropegia juncea* to treat various oral infections caused by bacterial pathogens.

Keywords: *Ceropegia juncea*, solvent extracts, phytochemicals, antibacterial activity, amikacin.

INTRODUCTION

Ceropegia juncea Roxb is a fleshy twining herb with tuberous roots. The genus *Ceropegia* belongs to the Asclepiadoideae (Milkweed) sub-family within the family Apocynaceae, is an important medicinal herb, which is used as a source of "Soma", a plant drug of the ayurvedic medicine with a wide variety of uses (Asolkar et al., 1992; Jagtap and Singh, 1999). The plant looks almost leafless as the leaves are minute, in opposite pairs and very sparsely distributed in the bare stem. Leaves are small, fleshy and lance-shaped. Flowers are borne in few-flowered umbels, large, goglet shaped, erect and beautifully variegated with yellow, green and purple. Mouth of the tube is much wider than other parts of the flowers. The united petals are light green in the lower half and dark green in the upper half [2, 3]. The fleshy stem of this plant is used as a raw material for traditional and folk medicines for the treatments of stomach and gastric disorders (Jain and Defillips, 1991). The decoction of the whole plant is used for the treatment of liver disorders and also in hypopotension, ulcerative condition and fever. The plant paste is topically used as an anaesthetic agent (Pavan Kumar et al., 2012). Stem of the *Ceropegia juncea* is crushed with milk and taken orally for three days. This stem paste is used to treat the ulcer (Karuppusamy, 2007).The alkaloid ceropegin was isolated and identified as pyridone type alkaloid, which is relatively rare in nature (Adibatti et al., 1991). The total alkaloidal fraction exhibited promising hepatoprotective, antipyretic, analgesic, local anesthetic, anti-ulcer, mast-cell stabilizing, tranquilising and hypotensive activities and was devoid of side effects. The present study was made to evaluate the antimicrobial property of solvent extract of this valuable plant.

MATERIALS AND METHODS

PLANT COLLECTION

Plant *Ceropegia juncea* was collected from different localities in and around Madurai and maintained in the herbal garden of Saraswathi Narayanan College (Autonomous), Madurai, Tamil Nadu, India. The plant was identified and authenticated by referring the standard taxonomic characteristic features (keys) according to the Flora of Madras Presidency (Gamble, 1935) and the Flora of Tamil Nadu Carnatic (Mathew, 1991). The voucher specimens of the plants and photographs were kept in the Department of Botany, Saraswathi Narayanan College (Autonomous), Madurai, Tamil Nadu, India for future reference.

PREPARATION OF EXTRACTS

The entire plant of *Ceropegia juncea* was used for preparation of extracts. The plant material was collected, cut into small pices, washed in water and dried at 40 ºC. The dried plant pieces were then grind in mechanical grinder. The powder was sieved using a mesh sieve and stored in air tight bottles. 50 g of the plant was taken in a Soxhlet's apparatus. The following series of solvents was used for the extraction: petroleum ether, chloroform, ethyl acetate and methanol. All solvents (250 ml) used were of analytical grade (AR). The extraction carried was of hot type and was for about 48 hours in each solvent. Before the successive solvent extraction, each time the powdered material was air dried below 50 ºC. To prepare aqueous extract fresh plant material (50 g) was harvested and used. The plant material was surface disinfected with 0.1 % (w/v) $HgCl_2$ solution for 5 min. It was washed thrice with sterilized distilled water for 5 min. each time. The plant material was ground with mortar and pestle in 100 ml sterile distilled water. The homogenized tissue was centrifuged at 3500 rpm for 20 min. The supernatant was taken as aqueous extract. All the extracts were concentrated by distillation of the solvent and evaporating to dryness using a flash evaporator. After complete solvent evaporation, each of the solvent extract was weighed and preserved at 5 ºC in air tight bottles until further use.

GROWTH AND MAINTENANCE OF TEST MICROORGANISMS

The Gram positive stains of Staphylococcus aureus and Streptococcus faecalis and Gram negative strains of Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris, Salmonella typhi, Chromobacterium violaceus and Burkolderia mallei were maintained at $37^{O}C$ in Nutrient Broth and the fungus Candida albicans was maintained in Potato dextrose agar medium until the preparation of inoculum.

INOCULUM PREPARATION

All the test bacterial species were maintained in nutrient agar media. 36 hours old bacterial cultures were inoculated into nutrient broth and incubated at 35 ± 2 ^{o}C on a rotary shaker (Remi, India) at 100 rpm. After 36 h incubation, the bacterial suspension was centrifuged at 10,000 rpm for 15 min. The pellet was resuspended in sterile distilled water and the concentration was adjusted to 1×10^8 cfu / ml using UV-visible spectrophotometer (Hitachi U-2000, Japan) by reading the OD of the solution to 0.45 (A_{610} nm) (Basri and Fan, 2005). Fungal colonies were harvested from 9-10 day old cultures, which were maintained on potato dextrose agar (PDA) medium. The spores were suspended in sterile distilled water and the spore suspensions were adjusted to 1×10^6 spores/ ml by counting with a haemocytometer (Dayang et al., 2005).

PHYTOCHEMICAL SCREENING

The qualitative phytochemical analyses were carried out following the Indian pharmacopoeia and the methods described by Harborne (1973). The five different solvent extracts obtained by successive solvent extraction were tested separately for the presence of various phytoconstituents namely alkaloids, amino acids, carbohydrates, fats and fixed oils, flavanoids, glycosides, saponins, gums, lignins, proteins, steroids, triterpenoids, tannins and phenolic compounds.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC values of the plant extracts were determined against the selected test organisms using the methods as described by National Committee for Chemical Laboratory Standard (1993). The extracts were added aseptically to sterile Mueller–Hinton

(MH) broth and potato dextrose agar (PDA) medium separately in appropriate volumes to produce the concentration range of 20-100 mg/ml. The resulting MH and PDA media were immediately poured into Petri plates after vortexing. The plates were inoculated with 100 µl of bacteria (1 x 10^8 cfu/ ml) and fungus (1x10^6 spores /ml) (Basri and Fan, 2005). Amikacin (20-30 µg) was used as a reference antibiotic drug and used as positive control. The paper discs loaded with 50 µl of DMSO and placed in MH and PDA were used as negative control. The inoculated plates were incubated at 37°C for 24 h and 37°C for 48 h for bacteria and the fungus respectively. At the end of the incubation period, the plates were evaluated for the presence or absence of growth of the test bacteria and fungus. The MIC values were determined as the least concentration of the extracts where absence of growth was recorded. For each extract, five replicates were maintained and each test was repeated at least thrice.

ANTIMICROBIAL ACTIVITY

In vitro antimicrobial activity was determined by using the agar disc diffusion method (Andrews, 2001). Whatman filter paper (No.1) discs of 6 mm diameter were impregnated with 50 µl of the solution of various extracts (at 60 mg /ml) prepared using DMSO. The discs were evaporated at 37°C for 24 hours. The standard antibiotics amikacin discs were prepared as described above using the appropriate concentration (30µg/ml) of the drug. An even spread of microorganisms was prepared by transferring 100 µl of microbial suspensions to Mueller-Hinton agar plates for bacteria (1 x 10^8 cfu / ml) and potato dextrose agar plates for the fungus (1 x 10^6 spores/ ml) using sterile cotton buds. The extract discs were then positioned on the inoculated agar surface. Standard 6 mm discs containing amikacin (30µg/ml) were used as positive controls. Negative controls were made using paper discs loaded with 50 µl of DMSO. The plates were then incubated at 37°C for 24 h for the bacteria. On the other hand, *Candida albicans* was incubated at 37°C for 48 h. The screening for antimicrobial activity was done by measuring the diameter of a clear inhibition zone around the disc. The mean diameter of inhibition zone was measured to the nearest millimeter (mm) based on three readings of the diameter zones of each target microorganism using a vernier caliper. For each extract, five replicates were maintained and each test was repeated at least thrice. Then the results were expressed as mean ± S.E.

RESULTS

PHYTOCHEMICAL SCREENING

The extracts of *Ceropegia juncea* were screened and their phytochemical constituents are presented. The phytochemical analyses exhibited differential occurrence of phytocompounds with respect to solvents used in this study. The solvent extractive values for solvent extracts were varying from 0.1% to 3.52%. The solvents used in this analysis revealed the maximum extractive value (3.52 %) while using methanol and followed by petroleum ether (2.13 %) water (2.054 %) and chloroform (1.39 %). The extractive value was comparatively very less in ethyl acetate (0.1 %). The phytochemical analysis of extracts exhibited that alkaloids and carbohydrates were found in all the solvent extracts used while saponin and steroids and sterols were detected in methanol and aqueous extracts only. The phytocompounds viz., triterpenoids, flavones and flavanoids, phenolic compounds, tannins and gum and mucilage were detected in the ethyl acetate extract. The presence of phenolic compounds and tannin also observed in the extracts prepared with chloroform and water. There was an absence of fixed oils and fat and glycosides in all the solvent extracts used (Table 1).

ANTIMICROBIAL ACTIVITY

The crude extracts of *Ceropegia juncea* exhibited MIC values between 20-80 mg/ml. (Table 2). MIC value of 20 mg/ml of ethyl acetate extract showed antimicrobial effect against all test organisms except *Pseudomonas aeruginosa* (40 mg/ml). Ethyl acetate extract had MIC value of 60 mg/ml against *Proteus vulgaris*, *Burkolderia mallei* and *Candida albicans*. In methanolic extract, 60 mg/ml of MIC was observed against *Proteus vulgaris* and *Candida albicans* while 80 mg/ml was noticed against *Salmonella typhi*. But, it was also effective at low MIC value of 20 mg/ml while tested against other organisms. Aqueous extract had MIC value of 20 mg/ml for all tested microorganisms except *Proteus vulgaris* and *Klebsiella pneumoniae* (40 mg/ml). Petroleum ether had lesser MIC value of 20 mg/ml against *Staphylococcus aureus* and *Escherichia coli*. But, 60 mg/ml was found to be potent against

Pseudomonas aeruginosa, *Proteus vulgaris*, and *Candida albicans*. Plant extracted with chloroform showed MIC of 20 mg/ml against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*. It was observed that extracts at 40-60 mg/ml were found to be MIC for most of the test microorganisms in this study. Antimicrobial activity of solvent extracts was compared with positive control (using amikacin for bacteria and ketokonazole for fungus) against Gram positive and Gram negative bacterial and a fungal microorganisms tested in this study. The aqueous extract had antimicrobial activity against all the tested organisms as compared to other solvent extracts. The extract was found to have significant effect against *Klebsiella pneumoniae* (18.05 mm) and *Proteus vulgaris* (17.05 mm). Poor inhibition zone was noticed against all test organisms while using petroleum ether and chloroform extracts. The ethyl acetate extract exhibited antimicrobial activity with the maximum zone of inhibition (16.95 mm) against one gram negative bacteria (*Pseudomonas aeruginosa*). But it was found to be lesser than the positive control (amikacin). The Gram negative bacterium viz., *Escherichia coli* (17.05 mm), *Pseudomonas aeruginosa* (17.05 mm) and *Klebsiella pneumoniae* (17.75 mm) were inhibited at significant level by methanolic extract when compared to positive control. The fungus *Candida albicans* was not inhibited by all solvent extracts of *Ceropegia juncea*. The results highlighted that significant inhibitory effect was found against *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* while using methanolic extract and *Klebsiella pneumoniae* and *Proteus vulgaris* in aqueous extract (Table 3 and Figure 1).

Table 1

Phytochemical screening of crude solvent extracts of Ceropegia juncea

	Phytochemical constituents										
Solvents used	**Alkaloids**	**Carbo Hydrate**	**Saponins**	**Tri terpenoids**	**Phenolic compounds & Tannins**	**Proteins & Amino acids**	**Steroids & Sterols**	**Fixed oil & Fat**	**Flavone & Flavanoids**	**Glycosides**	**Gum & Mucilage**
Petroleum ether	+	+	-	-	-	-	-	-	-	-	-
Chloroform	+	+	-	-	+	-	-	-	-	-	-
Ethyl acetate	++	+	-	++	++	-	-	-	+	-	+
Methanol	++	+	+	-	-	-	+	-	-	-	-
Water	+	+	+	-	+	-	+	-	-	-	-

+++ = maximum; ++ = moderate; + = minimum; - = absent.

Table 2

Minimum inhibitory concentration of crude solvent extracts of Ceropegia juncea

MIC (mg/ml)							
Test microorganisms	**Gram +/- Bacteria & Fungi**	**Solvent Extracts**				**Positive control**	
		Petroleum ether	**Chloroform**	**Ethyl acetate**	**Methanol**	**Water**	**Amikacin (µg/ml)**
Staphylococcus aureus	+	20.00	20.00	20.00	20.00	20.00	30
Streptococcus faecalis	+	-	80.00	20.00	-	20.00	20
Escherichia coli	-	20.00	20.00	20.00	20.00	20.00	30
Pseudomonas aeruginosa	-	60.00	60.00	40.00	20.00	20.00	25
Klebsiella pneumonia	-	80.00	20.00	20.00	20.00	40.00	25
Proteus vulgaris	-	60.00	60.00	-	60.00	40.00	30
Solmonella typhi	-	-	80.00	-	80.00	20.00	30
Chromobacterium violaceum	-	-	-	80.00	20.00	20.00	30
Burkolderia mallei	-	-	-	60.00	20.00	20.00	25
Candida albicans	Fungus	60.00	60.00	60.00	60.00	20.00	(Ketokonazole 50 µg/ml)

Values are the average of at least three determinations

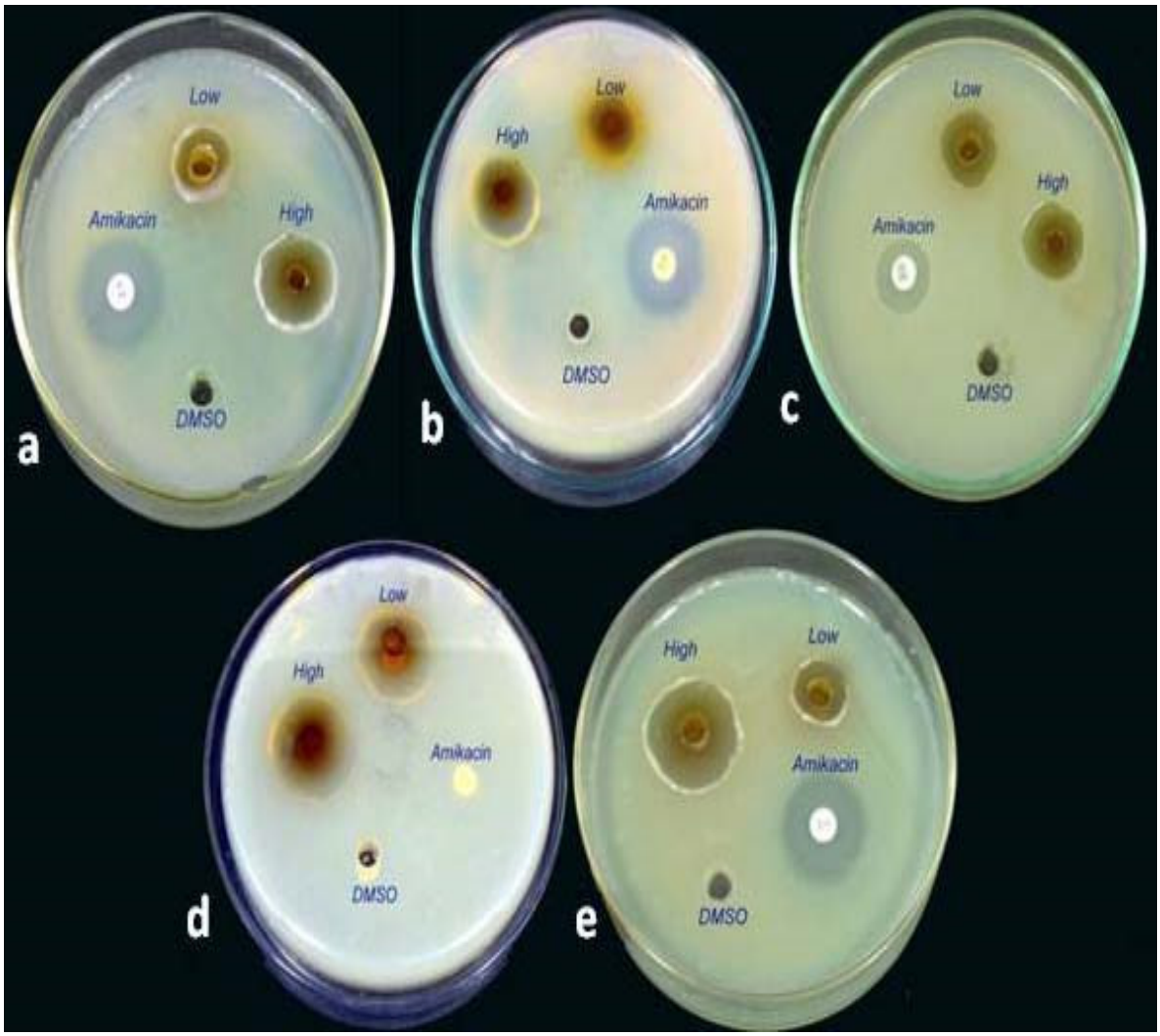
- = No inhibition

Table 3
Antimicrobial activity of crude solvent extracts of Ceropegia juncea

Zone of inhibition (mm)							
		Solvent Extracts			Positive control		
Test microorganisms	**Gram +/- Bacteria & Fungi**	**Petroleum ether**	**Chloroform**	**Ethyl acetate**	**Methanol**	**Water**	**Amikacin (30 µg/ml) / Ketakonazole (50 µg/ml)**
Staphylococcus aureus	+	03.96±0.10a	04.05±0.10bc	06.03±0.08b	09.95±0.10a	12.05±0.10b	17.03±0.10bb
Streptococcus faecalis	+	00.00±0.00a	03.08±0.10b	09.00±0.09b	00.00±0.00a	12.05±0.10b	19.02±0.08c
Escherichia coli	-	04.99±0.10b	04.97±0.09c	07.04±0.09b	18.05±0.10d	6.95±0.10a	18.90±0.09b
Pseudomonas aeruginosa	-	03.00±0.10ab	02.09±0.10a	16.95±0.10e	17.05±0.10cd	12.04±0.10 b	17.05±0.10b
Klebsiella pneumonia	-	01.17±0.10a	04.95±0.10c	05.04±0.09a	17.75±0.10cd	18.05±0.10e	17.95±0.10b
Proteus vulgaris	-	02.30±0.10a	03.06±0.00a	00.00±0.00a	04.75±0.10a	17.05±0.10e	16.95±0.09a
Solmonella typhi	-	00.00±0.00a	02.89±0.10a	00.00±0.00a	05.28±0.10a	13.05±0.10b	17.95±0.10b
Chromobacterium violaceum	-	00.00±0.00a	00.00±0.00a	04.12±0.10a	08.05±0.10bc	09.95±0.10a	19.05±0.10c
Burkolderia mallei	-	00.00±0.00a	00.00±0.00a	03.81±0.10a	06.95±0.10b	11.95±0.10b	20.06±0.10d
Candida albicans	Fungus	02.24±0.00a	01.99±0.10a	03.33±0.10a	06.09±0.10b	10.05±0.10ab	16.94±0.10a

Values represent mean ±SE. Mean followed by the same letter within columns are not significantly different (P=0.05) using Duncan's multiple range test.

Figure 1
Antimicrobial activity of crude solvent extracts of Ceropegia juncea



a, b & e – zone of inhibition of methanolic extract against Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli. c & d – zone of inhibition of aqueous extract against Klebsiella pneumoniae and Proteus vulgaris.

3. DISCUSSION

The solvents used for preparation of crude extracts of *Ceropegia juncea* exhibited maximum extractive value in methanol**.** In earlier studies, the high extractive value of methanol for woody stem extract of *Wrightia tinctoria* (Pritam and Sanjay, 2011) and ethanol for whole plant extract of *Cardiospermum halicacabum* (Viji and Murugesan, 2010) was reported. The phytochemical analysis of crude extracts of *Ceropegia juncea* revealed the presence of alkaloids in all solvent extracts. There was also a resemblance in occurrence of saponin and steroids in methanol and aqueous extracts. Similarly, phenolic compounds and tannins were found common in the extracts prepared with ethyl acetate, chloroform and water. Among the solvent extracts, ethyl acetate had a majority of phytoconstituents. The extracts prepared with petroleum ether and chloroform had least number of compounds, but they are biologically potential secondary metabolites. The observations on phytochemical screening are in accordance with the findings made in previous studies which reported variation in occurrence of bioactive compounds in different solvent extracts of whole plant of *Ceropegia* species

(Muthukrishnan et al., 2013). The differential occurrence of phytoconstituents recorded in this study can be rationalized in terms of polarity of the compounds. The predetermination of MIC of crude solvent extracts noticed that MIC value was ranging from 20 and 80 mg/ml irrespective of solvents. In a study, the MIC value was between 20 to 80 mg/ml for bacteria and fungi while using methanolic extracts of *Chelidonium majum* (Ciric et al., 2008) and it was ranging from 50 to 80 mg/ml for different methanolic extracts of stem, leaf and root of *Vitellaria paradoxa* (Ndukwe et al., 2007).

In *Ceropegia juncea*, the methanolic extract noticed significant inhibitory activity against Gram negative bacteria viz., *Escherichia coli* (18.05 mm), *Pseudomonas aeruginosa* (17.05 mm) and *Klebsiella pneumoniae* (17.75 mm) whereas *Klebsiella pneumoniae* (18.05 mm) and *Proteus vulgaris* (17.05 mm) alone found sensitive to aqueous extract of *Ceropegia juncea* when these extracts compared with positive control. The observations emphasized that methanolic and aqueous extracts of whole plant of *Ceropegia juncea* found to have an inhibitory effect against only few Gram negative bacteria. Similarly, the antibacterial efficiency of methanoic and aqueous extracts of *Gymnema sylvestre* was also reported against those test, Gram negative bacteria (Sripathi et al., 2010; Murugan and Mohan, 2012). Moreover, the study evidences that adult plant extracts of species were bacteriostatic at lower concentrations and bacteriocidal at higher concentrations. The antibacterial activity of *Ceropegia juncea* clearly reflects that it could be associated with phytocompounds such as alkaloids, phenolics, tannins, aminoacids, flavanoids, steroids, saponins and glycosides which were reported to be effective antimicrobial substances against wide range of microorganisms (Okwu and Okwu, 2004; Wani et al., 2012; David and Sudarsanam, 2013).

4. CONCLUSION

In the present study, the ethyl acetate and methanolic extracts found to have maximum number of phytoconstituents and followed by water. The qualitative analysis confirms that phytochemicals viz., alkaloids, phenolic compounds, tannins, triterpenoids, steroids, flavanoids and glycosides are found in the different solvent extracts of *Ceropegia juncea*. It was observed that MIC value varying with respect to test pathogens and solvents preferred. The difference in MIC values can be associated with qualitative and quantitative variations in phytocompounds assessed in the different solvents employed in preparation of extracts. The antibacterial activity focused that the methanolic extract found to have a significant inhibiting effect against *Pseudomonas aeruginosa, Escherichia coli, and Klebsiella pneumonia* and aqueous extract noticed the growth retarding effect for *Klebsiella pneumoniae* and *Proteus vulgaris*. It is observed that Gram positive bacteria were resistant to extracts used in this study. The results implicate the requirement of higher concentrations of the plant extracts have an inhibitory effect on other test organisms. It may be concluded that the adult dried parts of *Ceropegia juncea* might represent a new antimicrobial source with stable, biologically active components that can establish a scientific base for the use in modern medicine. However, further studies are needed to isolate and characterize the bioactive principle to develop new antimicrobial drugs from this species.

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