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PHYTOCHEMICAL, ANTIOXIDANT, ANTIBACTERIAL ACTIVITIES OF *NERVILIA CROCIFORMIS* (ZOLL. & MORITZI) SEIDENF. AND *NERVILIA INFUNDIBULIFOLIA* BLATT. & MC CANN (ORCHIDACEAE) COLLECTED FROM KASARGOD DISTRICT, KERALA, INDIA

P. K. Ruthisha *, C. Swathi Krishna and K. M. Khaleel

Department of PG Studies and Research in Botany, Sir Syed College, Taliparamba, Kannur - 670142, Kerala, India.

Keywords:

Phytochemicals, Medicinal plants, Antioxidants, Antimicrobial, Nervilia

Correspondence to Author:
P. K. Ruthisha

Department of PG Studies and Research in Botany, Sir Syed College, Taliparamba, Kannur - 670142, Kerala, India.

E-mail: ruthishapk@gmail.com

ABSTRACT: The whole plant extracts of *N. crociformis* and *N. infundibulifolia* was analyzed by priliminary phytochemical, total phenolics, flavonoid, carbohydrate and alkaloid content by standard methods. *In-vitro* antioxidant DPPH assay and Phosphomolybdenum assay developed by Braca *et al*., Prieto *et al*., respectively. *In-vitro* antibacterial (agar well diffusion) activity by gram negative *Pseudomonas aeroginosa* (ATCC 27853), gram positive (*Streptococcus mutans* MTCC 890 & *Staphylococcus aureus* ATCC 25923, *Esteritia coli* ATCC 25922) and antifungal activity by *Aspergillus niger* (ATCC 16404). The study shows that the petroleum ether, chloroform, ethanol and aqueous extract of the plants contains a good number of phytochemicals such as flavonoid, cardiac glycoside, saponin, alkaloid *etc*. as well as antioxidant with respect to the scavenging activity against free radicals DPPH which were roughly comparable to that of BHT. The plant extracts also exhibited potent antimicrobial activity against *S. aureus, P. aeruginosa, S. mutans, E. coli* and *A. niger* indicating its bactericidal and fungicidal properties. It may be concluded that the plant *Nervilia crociformis* is chemically more active than *Nervilia infundibulifolia*.

INTRODUCTION: India is a mega-biodiversity country, which is not only rich in medicinal plant resource, but also rich in traditional knowledge about such medicinal plants. There are several studies to prove the presence of various bioactivities and bioactive compounds in Orchidaeae plants. *Nervilia* is a genus of orchid with about 65 species among them 16 occurring in India.

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Among the 16 species *Nervilia aragoana* has subjected to various studies and the plant is commonly known as Orilathamara. But in many part of the country traditional healers use other species of *Nervilia* such as *Nervilia crociformis*, *Nervilia plicata* and *Habernaria diphylla* were used instead of *N. aragoana*.

In Sanskrit the drug Padmacarini is reported hot, bitter and astringent [1]. It overcomes morbid kapha and vatha and cures dysuria, urinary calculi, cough and asthma [2]. According to Rajanikhandu the drug is aromatic and it cures vomiting, epilepsy, diarrhoea, diabetis [3]. The Padmacarini is a constituent drug of Priyangvadi gana of Vagbhada. Vastyamayantaka ghrtam, Satavaryadi ghrtam and

Matsyakhyadi kashaya *etc*. are some of the preparations using the drug [4, 16]. Most of the literature has illustrated the padmacarini as *Hybanthus enneaspermus* and *Habernaria grandiflora*. Traditional healers and ayurvedic physicians of Kerala have considered the single leafed plants orilathamara is same as padmacarini and they used the plants *Nervilia crociformis, Nervilia plicata* and *Habernaria diphylla* as padmacarini. The present study aims at qualitative and quantitative phytochemical screening with special reference to the antioxidant and antibacterial properties of the plant *Nervilia crociformis* which is known as padmacarini in Sanskrit and a closely related species *Nervilia infundibulifolia* whole plant extracts.

Indian species of *Nervilia infundibulifolia* have not yet been subjected to such phytochemical screening to understand the medicinal values. The selected plants were belonging to the family Orchidaceae. The plants have a single leaf and are ground hugging in case of *N. crociformis* and small and with reddish patches in case of *N. infundibulifolia*. These plants have a fleshy flower stem with only one flower and a leaf which arises later at the base of the dying flower stem. The flowers of the plants appear soon after the first heavy rain in monsoon season.

MATERIALS AND METHODS:

Preparation of Extracts: The whole plants of *N. crociformis* and *N. infundibulifolia* were collected from Kasargod District, Kerala, India. The plants were identified in department of PG studies and research in botany, Sir Syed College, Taliparamba. The collected plants were washed thoroughly with tap water followed by distilled water for the removal of dust and soil particles, cut into pieces and shade dried at room temperature for 15 days then coarsely powdered using Panasonic Mixer and used for extraction. The powder (20 gm) was extracted with petroleum ether, chloroform, ethanol in a Soxhlet apparatus (3840; Borosil Glass works Ltd., Mumbai, India) and finally the dried powder was macerated using water with constant stirring for 48 h using the orbital shaker (Rivotek; Riviera Glass Pvt., Ltd., Mumbai, India) and the extract was filtered. The extracts were concentrated using a rotary evaporator and stored at -20 ºC in the deep freezer (RQV- 300; plus, REMI electro technik Ltd., Thane, Maharashtra, India) for further analysis. Quantitative analysis and antioxidant assays were done by dissolving 1 mg extract in 1 ml respective solvent. Antimicrobial study was done with well diffusion method by dissolving 1 mg extract in 1 ml DMSO.

Qualitative Phytochemical Screening: For preliminary phytochemical screening, plant extracts of the whole plants of *N. crociformis* and *N. infundibulifolia* was subjected to various qualitative chemical tests to determine the presence of phyto-constituents like glycosides, tannins, phytosterols, proteins, amino acids, carbohydrates, flavonoids, phenolic compounds, oils and fats, and saponins [5]. Total carbohydrate content of the selected plants was done by anthrone method [6]. The total phenolics of the different plant extracts were determined according to the method described by Makkar [7]. The flavonoid contents of all the extracts were quantified according to the method described by Zhishen, *et al*. [8]

***In-vitro* Antioxidant Assays:** Two different assays including free radical scavenging DPPH assay and Phosphomolybdenum assay were used to evaluate the antioxidant potential of the selected plants. Each experiment was done in triplicate and mean values were taken. The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH**,** according to the method of Braca *et al*. [9, 10] The antioxidant activity of samples was evaluated by the green phospho-molybdenum complex formation according to the method of Prieto *et al*. [11]

Antibacterial Activity Test: The antibacterial activity was carried out on the extracts chloroform and ethanol against gram negative (*Pseudomonas aeroginosa* ATCC 27853) and gram positive (*Streptococcus mutans* MTCC 890 & *Staphylococcus aureus* ATCC 25923), *Esteritia coli* (ATCC 25922) bacterial strains were used for the study. Antifungal activity by *Aspergillus niger* (ATCC16404).

The agar well diffusion method was employed for the determination of antimicrobial activities of the selected plants. A suspension of the tested bacteria (0.1 mL of 1×10^8 cells/mL) was spread on the

solid media plates. Wells of approximately 10 mm was bored using a well cutter and sample of 25, 50, and 100 µg concentrations were added. After staying at 4 ºC for 2 h, the plates were incubated at 37 ºC for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993) in centimetres. Streptomycin was used as a positive control with the tested bacteria. In order to access the biological significance and ability of the sample, the antifungal activity was determined by Agar well diffusion method. Potato Dextrose agar plates were prepared and overnight grown species of fungus, *Aspergillus niger* was swabbed. Wells of approximately 10 mm was bored using a well cutter and samples of different concentration was added; the zone of inhibition was measured after overnight incubation and compared with that of standard antimycotic (Clotrimazole).

RESULTS:

Phytochemical Screening: Screening of various phytochemicals of crude extracts reveals a good concentration of proteins, alkaloids, phenols, tannins, amino acids, carbohydrates, saponins, flavonoids, glycosides and fixed oils and fats in petroleum ether, chloroform, ethanol and water extracts of *Nervilia crociformis* **Table 1** and *Nervilia infundibulifolia* **Table 2**. These chemical compounds are responsible for different medicinal properties of the plant extracts.

TABLE 1: RESULTS OF PRELIMINARY PHYTOCHEMICAL SCREENING *N. CROCIFORMIS*

S. no.	Phytochemicals	Pet. ether	Chloroform	Ethanol	Water
1	Carbohydrate	+	++	+++	+++
2	Protein	+	+	+	++
3	Amino acid	-	-	+	+
4	Alkaloid	-	-	+	+
5	Flavonoid	+	+	+	+
6	Tannin	-	-	+	+
8	Saponin	-	-	-	+
9	Phenol	+	+	+	+
11	Cardiac Glycosides	-	-	+	-
12	Fixed oils and Fats	-	+	+	+

TABLE 2: RESULTS OF PRELIMINARY PHYTOCHEMICAL SCREENING *N. INFUNDIBULIFOLIA*

S. no.	Phytochemicals	Pet. ether	Chloroform	Ethanol	Water
1	Carbohydrate	+	++	+++	+++
2	Protein	-	-	+	+
3	Amino acid	-	-	-	+
4	Alkaloid	-	-	+	+
5	Flavonoid	+	+	+	+
6	Tannin	-	-	+	-
8	Saponin	-	-	-	+
9	Phenol	+	+	+	+
11	Cardiac Glycosides	-	-	-	+
12	Fixed oils and Fats	-	-	+	-

(+): Presence of chemical compound, (-): Absence of chemical compound; (+) < (++) < (+++): Based on the intensity of characteristic colour

Quantification of Total Carbohydrates:

TABLE 3: CARBOHYDRATE CONTENT IN THE SELECTED PLANT SAMPLES

Plant sample	Carbohydrate
Nervilia crociformis	152.93±.70
Nervilia infundibulifolia	311.53±5.29

Total carbohydrate content was high in *N. infundibulifolia* compared to *N. crociformis* and it was 311.53 ± 5.29 and in *N. crociformis* it was 152.93 ± .70.

Quantification of Total Phenolics: The phenolic content in the selected plants were studied by spectroscopic method. The phenolic content of the whole plant extract of *N. crociformis* and *N. infundibulifolia* in different solvent extracts were shown in **Table 4**.

TABLE 4: PHENOLIC CONTENT IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS

S. no.	Extract	Phenolics (GAE/1000mg)	
		N. c	N. i
1	Petroleum ether	24.28±.37	10.53±.44
2	Chloroform	17.23±.51	8.45±.27
3	Ethanol	60.49±2.03	27.43±.32
4	Water	23.53±.82	21.03±19

The chloroform extract shows lowest activity of the two species studied and the ethanol extract shows highest activity. Of the two plants studied the whole plant extracts of *N. crociformis* has highest phenolic content than *N. infundibulifolia*.

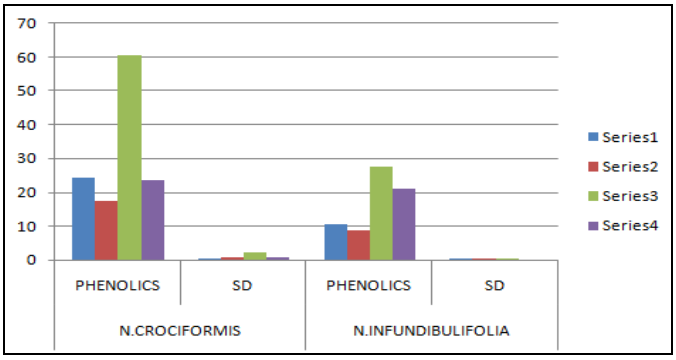


FIG. 1: GRAPHICAL REPRESENTATION OF TOTAL PHENOLICS IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS

Series 1; Pet. ether, Series 2; Chloroform, Series 3; Ethanol, Series 4; Water

Quantification of Total Flavonoids:

TABLE 5: TOTAL FLAVONOID CONTENT IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS

S. no.	Extract	Flavonoids (RE/1000mg)	
		N. c	N. i
1	Petroleum ether	115.49±.96	178.06±2.55
2	Chloroform	128.65±.56	147.26±1.92
3	Ethanol	124.79±1.47	128.01±4.41
4	Water	114.85±3.09	142.77±2.94

N. i: *Nervilia infundibulifolia*; N. c: *Nervilia crociformis*

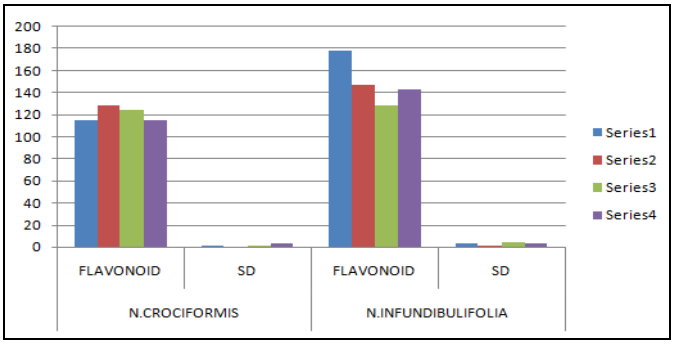


FIG. 2: GRAPHICAL REPRESENTATION OF TOTAL FLAVONOID IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS

Series 1; Pet. ether, Series 2; Chloroform, Series 3; Ethanol, Series 4; Water

Comparatively the plant *N. infundibulifolia* extracts may contain more flavonoid content than *N. crociformis* extracts.

Antioxidant Assays: In present study, the 4 extracts of *N. crociformis* and *N. infundibulifolia* were investigated for antioxidant potential by using three different methods. All extracts showed antioxidant activity up to varying extent.

DPPH Radical Scavenging Activity:

TABLE 6: DPPH RADICAL SCAVENGING ACTIVITY OF *NERVILIA CROCIFORMIS* AND *NERVILIA INFUNDIBULIFOLIA*

S. no.	Extract	IC_{50} mg/ml	
		N. c	N. i
1	Petroleum ether	48.16956	136.612
2	Chloroform	25.56237	88.65248
3	Ethanol	79.36508	111.1111
4	Water	24.11963	173.6111

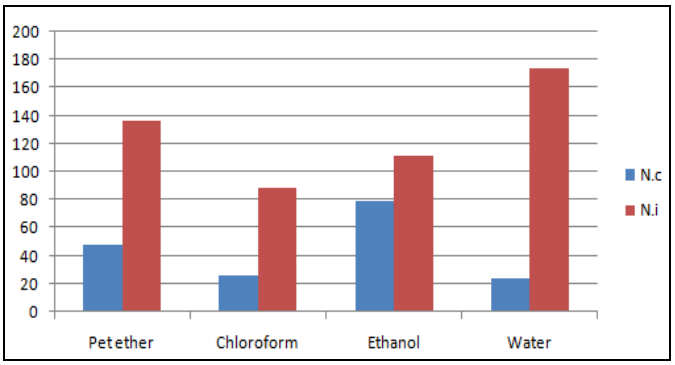


FIG. 3: GRAPHICAL REPRESENTATION OF DPPH FREE RADICAL SCAVENGING ACTIVITY IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS.

N. i: *Nervilia infundibulifolia*; N. c: *Nervilia crociformis*

The petroleum ether extract of *Nervilia crociformis* whole plant sample exhibit a significant dose dependent inhibition of DPPH activity with 50% of inhibition (IC_{50}) at concentration of 48.16956 mg/ml and IC_{50} value of BHT was 36.79176 mg/ml. The chloroform extract shows 50% inhibition at the concentration 25.56237 mg/ml, ethanol extract inhibit the free radical scavenger at a concentration of 79.36508 mg/ml and water extract shows an IC_{50} value of 24.11963 mg/ml.

In the case of *Nervilia infundibulifolia* the chloroform extract shows the lowest value and the chloroform extract of the selected plant was a good radical scavenger than all other extract and the concentration was 88.65248 mg/ml. The petroleum ether extract of *Nervilia infundibulifolia* whole plant sample exhibit 50% inhibition at the concentration 136.612, the ethanol extract shows an IC_{50} value of 111.1111 and water extract shows 50% of inhibition (IC_{50}) at concentration 173.6111.

Based on the demonstrated results the crude extract of *Nervilia crociformis* can be categories as more

free radical scavenging activity than *Nervilia infundibulifolia*. Basically, a higher DPPH radical-scavenging activity is associated with a lower IC_{50} value.

Phosphomolybdenum Assay: The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto (1999). This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour.

TABLE 7: RESULT OF PHOSPHOMOLYBDENUM ASSAY

S. no.	Extract	N.i	N.c
1	Petroleum ether	209.44±2.55	191.67±1.5
2	Chloroform	183.7±6.12	348.52±1.2
3	Ethanol	264.26±1.7	154.63±3.9
4	Water	325.93±1.4	293.89±6.3

N. i: *Nervilia infundibulifolia*; N. c: *Nervilia crociformis*

The petroleum ether extract shows an average of 209.44 ± 2.55, chloroform extract of *Nervilia infundibulifolia* shows an average of 183.7 ± 6.12, ethanol extract shows a value of 264.26 ± 1.7 and water extract shows a result of 325.93 ± 1.4. *Nervilia crociformis* shows an average of 191.67 ± 1.5 in petroleum ether extract, 348.52 ± 1.2 in chloroform extract, 154.63 ± 3.9 in ethanol extract and 293.89 ± 6.3 in water extract.

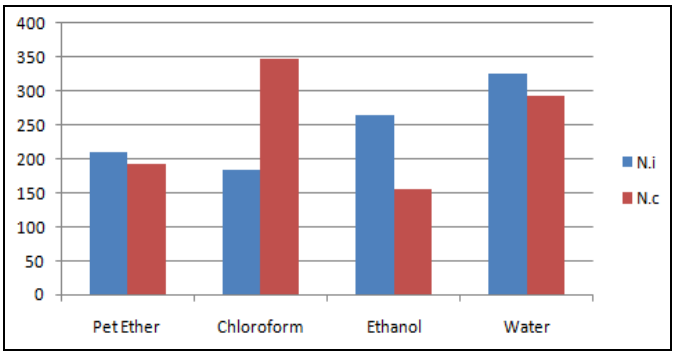
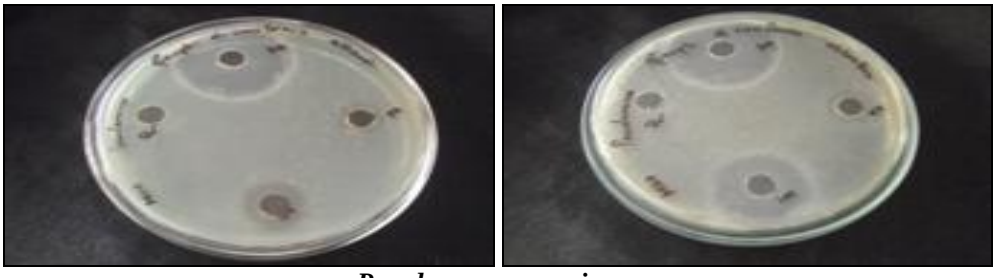


FIG. 4: GRAPHICAL REPRESENTATION OF PHOSPHO-MOLYBDENUM ASSAY IN *N. CROCIFORMIS* AND *N. INFUNDIBULIFOLIA* WHOLE PLANT EXTRACTS. N. i: *Nervilia infundibulifolia*; N. c: *Nervilia crociformis*

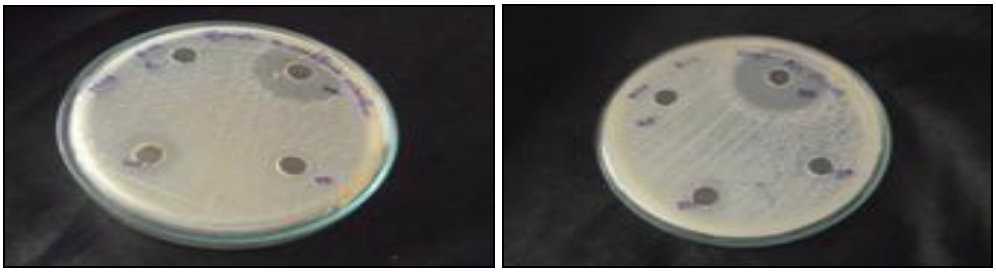
Antimicrobial Activity: Antimicrobial activity is observed high in chloroform and ethanol extracts compared to petroleum ether and water.

Streptococcus mutans

Staphylococcus aureus



Pseudomonas aeruginosa



Escheritia coli

Aspergillus niger

FIG. 5: ANTIMICROBIAL ACTIVITY OF *N. CROCIFORMIS* ETHANOL AND CHLOROFORM EXTRACTS AGAINST DIFFERENT STRAINS

TABLE 8: ZONE OF INHIBITION OF *N. CROCIFORMIS* CHLOROFORM AND ETHANOL EXTRACT AGAINST DIFFERENT BACTERIAL STRAINS

Sample	Concentration (µg)	Zone of inhibition (cm)			
		E. coli	*P. aeroginosa*	*S. aureus*	*S. mutans*
chloroform	25	1.0	nil	nil	1.0
	50	1.1	1.4	1.0	1.0
	100	1.6	2.3	1.5	1.3
ethanol	25	Nil	Nil	Nil	1.0
	50	1.0	1.2	nil	1.4
	100	1.4	1.7	1.3	2.0
Streptomycin	50	3.0	3.7	3.0	2.7

TABLE 9: ZONE OF INHIBITION OF *N. CROCIFORMIS* CHLOROFORM AND ETHANOL EXTRACT AGAINST *A. NIGER*

Sample	Concentration (µg)	Zone of inhibition (cm)
chloroform	25	nil
	50	1.0
	100	1.3
ethanol	25	Nil
	50	nil
	100	1.4
Clotrimazole	50	2.0

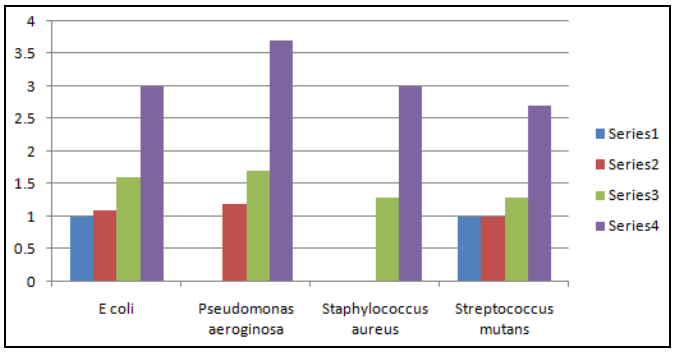


FIG. 6: GRAPHICAL REPRESENTATION OF ANTIMICROBIAL ACTIVITY AGAINST DIFFERENT BACTERIAL STRAINS IN CHLOROFORM EXTRACT

Series 1 concentration 25 µg, Series 2 50 µg, Series 3 100 µg, Series 4 antibiotics

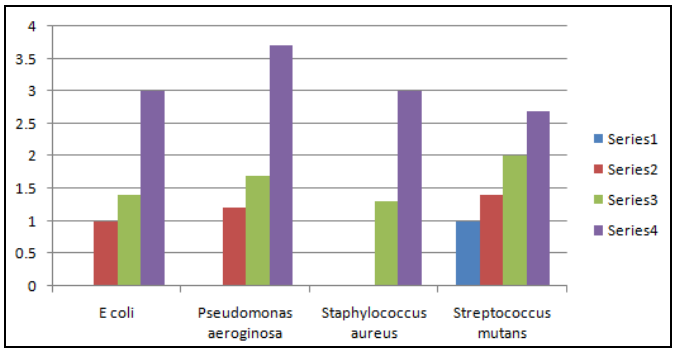


FIG. 7: GRAPHICAL REPRESENTATION OF ANTIMICROBIAL ACTIVITY AGAINST DIFFERENT BACTERIAL STRAINS IN ETHANOL EXTRACT

Series 1 concentration 25 µg, Series 2 50 µg, Series 3 100 µg, Series 4 antibiotics

The present study investigated the *in-vitro* antibacterial and antifungal activity of two different solvent extracts in *Nervilia crociformis* whole plant sample against four different strains of bacteria like *E. coli*, *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *Streptococcus mutans* and a fungal strain *Aspergillus niger*. The potency of the extracts and their antibacterial sensitivity were assessed quantitatively by determining the IZs given in **Table 8** and the antifungal activity were assessed and IZs given in **Table 9**.

In the case of antibacterial activity, the average IZ of the chloroform extract were highest against the growth of *Pseudomonas aeroginosa* and least in *staphylococcus aureus*. The average IZ of the ethanol extract were highest against the growth of *streptococcus mutans* and least in *Staphylococcus aureus*. The inhibition zone against different bacterial strains in ethanol and chloroform extracts shows in the **Table 8 Fig. 7** and **8**. Antifungal activity of the two extracts was shown in **Table 9**.

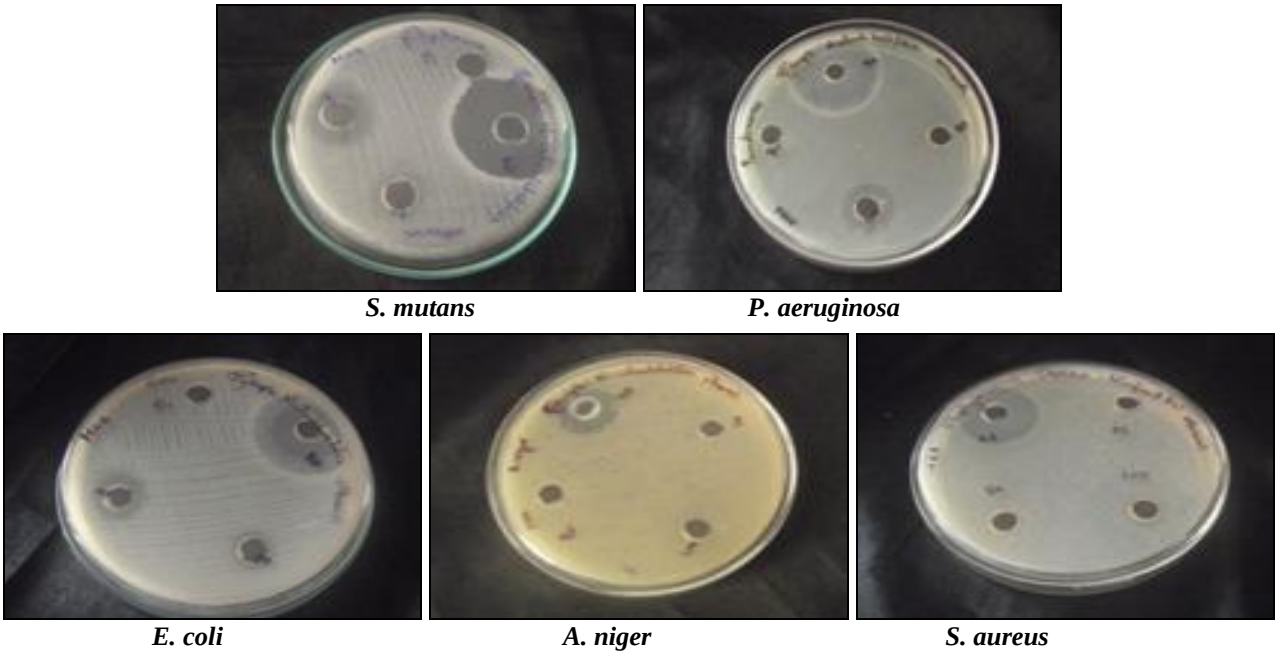


FIG. 8: ANTIMICROBIAL ACTIVITY OF *N. INFUNDIBULIFOLIA* ETHANOL EXTRACT AGAINST DIFFERENT STRAINS

TABLE 10: ZONE OF INHIBITION OF *N. INFUNDIBULIFOLIA* ETHANOL EXTRACT AGAINST DIFFERENT BACTERIAL STRAINS

Sample	Concentration (µg)	Zone of inhibition (cm)				
		E. coli	*P. aeroginosa*	*S. aureus*	*S. mutans*	A. niger
Ethanol	25	1.0	Nil	Nil	Nil	Nil
	50	1.2	Nil	Nil	1.2	Nil
	100	1.7	1.6	1.1	1.6	1.1
Antibiotics	50	3.0	3.7	3.0	2.7	2

The results as diameter of the inhibition zone in ethanol extract have been given in the **Table 10** and **Fig. 9**. The chloroform extract of the plant shows little activity against selected microbial strains so the results would be discarded. IZ of *N. infundibulifolia* whole plant ethanol extract is maximum in the case of *E. coli* and minimum in *S. aureus*.

Streptomycin was used as control in the case of antibacterial activity and Clotrimazole in the case of antifungal activity.

DISCUSSION: Many species of orchids having helpful phytoconstituents are currently being used as drugs in the Indian system of medicine. Being members of a highly advanced family, orchids have a major role to play in the genetic engineering of new forms that may be useful in floriculture, pharmacology and other, as yet unexplored fields of science. Classical texts highlighted that orchid drugs like Jeevaka, Rishabhaka, Riddhi as Rasayana [12] and Vajeekarana agents [13] and used mainly in the treatment of Vata dominant diseases (which involves nervous system and musculo-

skeletal disorders mainly) [13]. Certain conditions like rheumatism, fractures, ear ache, nervous disorders, wounds, sexual problems, swellings, worm infestation, headache are found to be managed by the traditional practitioners with simple formulations consisting of orchids.

The results obtained from the present study provide certain informations with respect to the phyto-chemical constituents, antioxidant property, antibacterial activity of the two species of medicinal orchid Nervilia namely *Nervilia crociformis* and *Nervilia infundibulifolia*. These two species are closely related in their morphology and characteristics. In this study the authors try to identify the differences in chemical properties of these two species because these two species were used interchangeably for a certain disease kidney stone.

As evident from the phytochemical analysis **Table 1** and **2**, Out of the 12 phytochemicals studied ethanol and water extracts of *Nervilia crociformis* contains a good number of phytochemicals compared to *Nervilia infundibulifolia*. But quantitatively carbohydrate and flavonoid content was high in *Nervilia infundibulifolia.* Surprisingly all the extracts of *Nervilia infundibulifolia* studied show good result than that of *Nervilia crociformis.* In the case of total phenolic content the case was just reversed, *Nervilia crociformis* shows good results than *Nervilia infundibulifolia* in all extracts.

The results of free radical scavenging activity of the plants shows that *Nervilia crociformis* extracts were good free radical scavenger compared to *Nervilia infundibulifolia*. The presence of free radical scavenging property in high amount in the plant extract supported by its rich phytochemicals that imparts antioxidant activity contributes to curing of various diseases [14, 15]. The free radical scavenging property of present observation may be attributed to the alkaloid, saponin and cardiac glycoside which were detected in conspicuous amount [16].

The plant extracts in ethanol and chloroform shows significant antimicrobial activity in the case of *Nervilia crociformis* and chloroform extract of *Nervilia infundibulifolia* shows little activity against the studied strains.

Out of five test strains, all the strains namely *S. aureus* (gram +ve, inhabit the respiratory tract is a facultative pathogen) and *P. aeruginosa*, (gram -ve, cause infection in blood and lungs), *E. coli* (gram -ve, intestinal infection and fever), *S. mutans* (gram +ve, inhabiting in the mouth of humans) were found to be inhibited by the 100 µg ethanol and chloroform extract of *Nervilia crociformis* and ethanol extract of *Nervilia infundibulifolia*. The results obtained were found to be encouraging as compared to that of Streptomycin. *A. niger* was the fungal strain studied and the results were satisfactory in the case of the extracts of the two studied plants.

CONCLUSION: Hence, orchids were commonly used as traditional medicine many of the medicinal orchids were yet to be explored for their medicinal properties. In the present study indicated that *Nervilia crociformis* and *Nervilia infundibulifolia* two medicinal orchids possess a good phyto-chemical, antibacterial and antioxidant activities. Further, work is required to find out the active principle from the plant extracts and to carry out pharmaceutical studies.

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CONFLICT OF INTEREST: Nil

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