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Research Article

EVALUATION OF BIOACTIVE POTENTIAL OF *COELOGYNE NERVOSA* A.RICH. - AN ENDEMIC MEDICINAL ORCHID OF WESTERN GHATS, INDIA

SAHAYA SHIBU B[1]. CHITRA DEVI B[2*]. SARMAD MOIN[1]. SERVIN WESLEY P[1]

[1]Department of Biotechnology, Karpagam University, Coimbatore, India,[2]Department of Botany, Karpagam University, Coimbatore, India,Email: chitrasaraswathy@gmail.com

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ABSTRACT

The study has been designed to examine the phytochemical, antimicrobial, antioxidant and anticancer activity of *Coelogyne nervosa* A.Rich. leaf extracts. Preliminary phytochemical screening for the presence of alkaloids, carbohydrates, glycosides, saponins, terpenoids, steroids, flavonoids, phenolic compounds, protein, gum and mucilages, phytosterol, tannins and phlobatannins were carried out. The antimicrobial, antioxidant and anticancer activity were also performed. Preliminary phytochemical analysis revealed that the ethanol and aqueous extracts shown the maximum phytochemical constituents. All the extracts of *Coelogyne nervosa* were tested for antimicrobial and antioxidant activities. The ethanol extract showed the maximum antibacterial activity against all the microorganisms tested, which shows the MIC value in the range between 400-600 (µg/ml). The water extract possessed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} 126 µg/ml) and it shows cytotoxic activity towards the MCF-7 cancer cell line (IC_{50} 292.8 µg/ml).These findings states that the *Coelogyne nervosa* exhibits potential antimicrobial, antioxidant and anticancer properties.

Keywords:

INTRODUCTION

Orchidaceae is highly evolved and widely distributed monocotyledonous family with large number of terrestrial, saprophytic and epiphytic species. It comprises more than 30, 000 species in 750 genera [1]. They are known for their diversity of habitats, and they occur in diverse habitat conditions of our country[2]. Orchids have tremendous scope in horticulture and pharmacognosy, five *Dendrobium* species are included in the Chinese pharmacopoeia. They are assumed to be effective in some diseases or syndromes related to the deficiency of yin in the kidney, lung and stomach. It is also used as the remedies for fever, red tongue, atrophic gastritis and diabetes[3] but this potential has remained largely untapped in India[4]. Many orchids were used in Chinese traditional medicine as a remedy for a number of treatments. The first record of Indian orchids used in ayurvedic medicine is *Eulopia dabia* (D. Don) Hochr., *Flickingeria nodosa* (Dall) and *Malaxis rheedii* SW., which was discussed in 'Charaka Samhita', a classic ancient Indian medicinal treatise written by Charaka in Sanskrit, a few thousand years ago[5]. *Microstylis walliachii* is used in treatment of tuberculosis and the juice of *Dendrobium ovatum* is helpful in all kinds of stomachache, bile secretion and is used as a laxative [6]. Leaves of *Dendrobium nobile* and *Cleisostoma williamsonii* is used in wounds for quick healing and for bone fracture whereas, *Eulophia nuda* tuber extracts are used for blood purification. The seeds of *Cymbidium madidum* and pseudobulbs of *Dendrobium* spp. used as oral contraceptive [7]. *Coelogyne stricta* is used in folk and tribal medicines as a cure for bone fracture, fever, and headache [8]. The tubers and pseudobulbs of several orchids like *Orchis latifolia, Orchis mascula, Cymbidium aloifolium, Zeuxine strateumatica,* and some species of *Dendrobium, Eulophia* and *Habenaria* are used as a restorative and in the treatment of various diseases[9]. The powdered roots of *Vanda tessellata* is considered as antidote for poisoning and also in rheumatic pains and abdominal complaints. *Dendrobium fimbriatum* has been used for liver upsets and nervous debility, while *Dendrobium teretifolium* for headache and pain reliever in other parts of body. Other orchid genera like *Oberonia*, *Eria, Bulbophyllum, Eulophia, Geodorum, Grammatophylum*, and *Hetaeria* are also reported to be used as medicine in different parts of the world to cure various diseases[10,11].

Coelogyne nervosa is an endemic orchid of Western Ghats which grows as an epiphyte and is important for its beautiful flowers [12]. The *in vitro* asymbiotic seed germination of *Coelogyne nervosa* has been already reported [13]. Hence the present study reports the preliminary phytochemical, antimicrobial, antioxidant and anticancer potential of the orchid *Coelogyne nervosa* for the first time.

MATERIALS AND METHODS

Plant material

Coelogyne nervosa, leaves were obtained from the National Orchidarium & Experimental Garden, Botanical Survey of India (Southern Regional Centre), Yercaud, India.

Preparation of plant extracts

The *Coelogyne nervosa* leaves were washed and shade dried, then ground into fine powder. The extraction was carried by soxhlet extraction technique. Different solvents were used successively with gradient polarity (petroleum ether, chloroform, ethyl acetate, ethanol and water). The colour and percentage yield were noted. The extracts were completely evaporated by vacuum distillation and stored.

Phytochemical screening

Preliminary phytochemical screening was carried out according to the methods described by Harborne,[14] Trease and Evans[15] and Raaman[16].

Test Microorganisms

The test organisms included *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*), *Bacillus subtilis* MTCC 2393 (*B. subtilis*), *Salmonella enterica* typhimurium MTCC 98 (*S. enterica*) and *Corynebacterium* spp. MTCC 3080 (*Corynebacterium* spp.). All the strains were collected from American Type Culture Collection, Manassas, USA and Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The microorganisms were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C.

Disc diffusion method

Coelogyne nervosa leaf extracts were screened for antimicrobial activity using disc diffusion method[17]. 18hrs old cultures of microorganisms maintained in Potato Dextrose Broth (Hi-media, Mumbai) were used. Sterilized discs (Hi media, 6 mm), soaked in a known concentration of the crude extracts of *Coelogyne nervosa* (500µg/ml DMSO of per disc). Soaked discs were applied over each of the culture plates previously seeded with the 0.5 McFarland. Antibiotic discs of Chloramphenicol (30mcg/disc) were used as positive control and paper discs with DMSO were used as negative

control and incubated at 37°C for 24 - 48hrs. Zones of inhibition were measured, and the mean diameter was recorded.

Determination of Minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration was carried out according to the methods of [18] with little modification. A stock concentration 1mg/ml of the extract was prepared. From the stock, 50, 100, 150, 250, 500, 750 and 1000µg of each concentration were added to each 9ml of nutrient broth containing 0.1ml of standardized test organisms. The tubes were incubated at 37°C for 24hrs. A positive control was equally set up by using DMSO and test organisms without extract. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration.

Antioxidant activity

All the five extracts of *Coelogyne nervosa* and ascorbic acid were dissolved in 100% dimethyl sulfoxide (1mg/ml) separately and used for the *in vitro* antioxidant assay. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity based on[19] and ferric ion reducing antioxidant power (FRAP) assay according to Benzie and Strain[20] were analysed to investigate the antioxidant properties.

***In vitro* anticancer activity**

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma) assay[21] with little modification. The human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37^0C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. 100µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48hrs at 37^0 C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48hrs of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570nm using micro plate reader. The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using GraphPad Prism software.

RESULTS

Preliminary Phytochemical analysis

The maximum percentage yield was observed in petroleum ether extract (6.91%), and minimum in ethyl acetate extract (2.96%). Colour changes recorded was Dark green to Dark brown in petroleum ether to distilled water (Table 1). Preliminary phytochemical screening of *Coelogyne nervosa* revealed the presence of alkaloids, carbohydrates, glycosides, saponins, terpenoids, steroids, flavonoids, phenolic compounds, proteins, gum and mucilages, phytosterols, tannins and phlobatannins, while it gave negative results to amino acids, anthroquinones, fats and oils and carotenoids. Of this ethanol extract shows the presence of maximum phytochemical constituents followed by water extract (Table 2). These Phytochemicals present in the leaf of *Coelogyne nervosa* shows that the plant have more pharmacological importance.

Table 1:Percentage yield and colour of the extracts of *Coelogyne nervosa* in various solvents.

Extracts	Percentage yield (W/V)	Colour
Petroleum ether	6.91 %	Dark green
Chloroform	5.61 %	Dark green
Ethyl acetate	2.96 %	Light green
Ethanol	4.33 %	Brown
Aqueous	4.59 %	Dark brown

Table 2:Preliminary phytochemical analysis of *Coelogyne nervosa* leaf extracts

S. No	Phytochemical tests	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Aqueous
1.	Alkaloids	-	-	+	+	+
2.	Carbohydrates	-	+	-	+	+
3.	Glycosides	-	-	+	+	+
4.	Saponins	-	-	-	+	-
5.	Terpenoids	+	-	+	+	+
6.	Steroids	+	+	-	-	-
7.	Flavonoids	-	-	-	+	+
8.	Phenolic compounds	-	+	+	+	+
9.	Protein	+	+	-	-	-
10.	Amino acids	-	-	-	-	-
11.	Anthroquinones	-	-	-	-	-
12.	Fats and oils	-	-	-	-	-
13.	Carotenoids	-	-	-	-	-
14.	Gum and Mucilages	+	+	-	-	-
15.	Phytosterol	+	+	+	+	+
16.	Tannins	-	-	-	+	+
17.	phlobatannins	-	-	-	+	+

Where + Present, - Absent

Antibacterial screening

The antibacterial activity of the leaf extracts of *Coelogyne nervosa* was presented in the table 3. Of the five microbes tested (*Pseudomonas aeruginosa*, *Enterococcus facealis*, *Bacillus subtilis*, *Salmonella enteria*, *Corynebacteria* spp.) all the microbes were susceptible to the leaf extract of *Coelogyne nervosa* which includes three Gram-positive bacteria (*Enterococcus facealis*, *Bacillus subtilis*, *Corynebacteria* spp.) and two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Salmonella enteria*). The ethanolic extract of *Coelogyne nervosa* showed the maximum zone of inhibition against the bacteria *Pseudomonas aeruginosa* (15 mm) followed by *Enterococcus facealis* (14.3 mm) and *Salmonella enteria* (12 mm), whereas *Bacillus subtilis*, *Corynebacteria* spp. had 11 and 9 mm zone respectively.

Table 3:Antibacterial activity of *Coelogyne nervosa* leaf extracts

S.no	Organism	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Aqueous	Positive control (Chloramphenicol)
1	*Pseudomonas aeruginosa* (ATCC 27853)	12 mm	14 mm	9 mm	15 mm	-	32 mm
2	*Enterococcus facealis* (ATCC 29212)	-	12.3 mm	9.6 mm	14.3 mm	-	36 mm
3	*Bacillus subtilis* (MTCC 2393)	-	-	9 mm	11 mm	-	33 mm
4	*Salmonella enteria* (MTCC 98)	9 mm	10 mm	11mm	12 mm	-	29 mm
5	*Corynebacteria* spp. (MTCC 3080)	8 mm	-	10 mm	9 mm	8.3 mm	32 mm

Minimum Inhibitory Concentration

Minimum Inhibitory Concentration of the ethanol extract of *Coelogyne nervosa* showed the strongest antibacterial activity with MIC value of 0.400mg/ml against *Enterococcus faecalis* followed by *Pseudomonas aeruginosa* (0.450mg/ml) and *Salmonella enterica* (0.500 mg/ml) (Table 4). Whereas the MIC value for *Corynebacterium* spp. and *Bacillus subtilis* was 0.550mg/ml and 0.600mg/ml, respectively.

Table 4:Minimum Inhibitory Concentrations of ethanol extract of *Coelogyne nervosa*

S. No.	Organisms	Minimum Inhibitory Concentrations (µg/ml)
1	*Pseudomonas aeruginosa* (ATCC 27853)	450
2	*Enterococcus facealis* (ATCC 29212)	400
3	*Bacillus subtilis* (MTCC 2393)	600
4	*Salmonella enteria* (MTCC 98)	500
5	*Corynebacteria* spp. (MTCC 3080)	550

Antioxidant activity

The IC_{50} values of all the extracts of *Coelogyne nervosa* were calculated from the logarithmic regression curve. The IC_{50} values of DPPH radical scavenging activity of various extracts were shown in the figure 1. The best free radical (DPPH) scavenging activity was observed in the aqueous extract of *Coelogyne nervosa* leaves with IC_{50} value 126µg/ml, followed by the ethanol and ethyl acetate extract (IC_{50} value 206µg/ml and 312µg/ml respectively). The ferrous reducing ability of various extracts of *Coelogyne nervosa* leaves were in the range of 130.59–499.65µM of AAE/g Dry weight (Table 5).

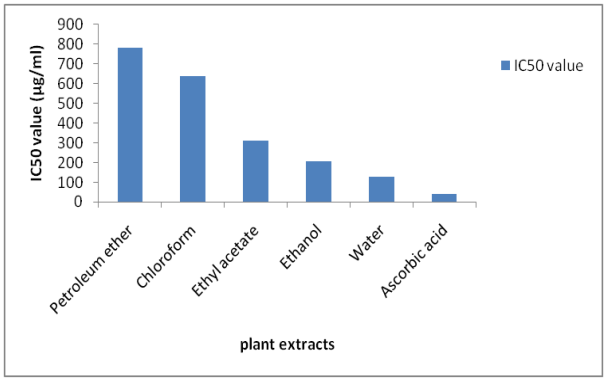

Figure 1:DPPH radical scavenging activity of *Coelogyne nervosa* extracts.

Table 5:FRAP activity of *Coelogyne nervosa* extracts

Extracts	FRAP (µM of AAE /g Dry weight)
Petroleum ether	130.59 µM
Chloroform	181.69 µM
Ethyl acetate	227.11 µM
Ethanol	238.47 µM
Aqueous	499.65 µM

Cytotoxic activity

The anticancer activity of the aqueous extract of *Coelogyne nervosa* leaves were investigated using the human breast cancer cell line (MCF-7). The aqueous extract of *Coelogyne nervosa* leaves against the MCF-7 was shown in the Figure 2 and Table 6. The IC_{50} value of aqueous extract on MCF-7 cell line was 292.8µg/ml. This was the first report on *Coelogyne nervosa* against the anticancer activity.

Table 6:Percentage cell inhibition of aqueous extract of *Coelogyne nervosa* leaves on MCF-7 cell line.

Concentration (µg)	% cell inhibition
18.75	0.16 %
37.5	3.99 %
75	6.85 %
150	28.95 %
300	59.67 %

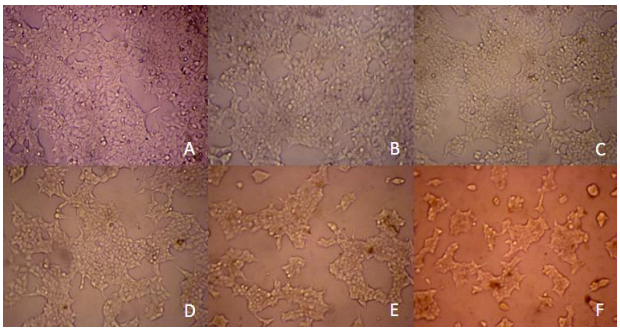

Figure 2:Cytotoxic activity of aqueous extract of *Coelogyne nervosa* leaf on MCF-7 cell line.

Where, A-Control, B-18.75µg, C-37.5µg, D-75µg, E-150µg, F-300µg

DISCUSSION

Numerous orchid species are traditionally used as herbal medicine as a remedy for microbial infections and many other ailments, but the potential of most of the orchid species for therapeutic use is yet to be scientifically explored [22]. Alkaloids are nitrogenous organic heterocyclic molecules that have pharmacological effects on humans and other animals. They are secondary metabolites of plants and the well-known alkaloids include strychnine, morphine, codeine, nicotine, atropine, cocaine, quinine, methamphetamine, reserpine, caffeine and theophylline. In orchids, 214 species in 64 genera contain 0.1% or more alkaloids. In China, 8% of *Dendrobium* species, 18% of *Eria* species and 42% of *Liparis* species have this degree of alkaloid content [23]. The phytochemical analysis shows the presence of alkaloids, carbohydrates, glycosides, saponins, terpenoids, steroids, flavonoids, phenolic compounds, protein, phytosterol, tannins and phlobatannins in ethanol extract. This result is similar to the results of Sarmad *et al*,[8] and Mazumder *et al*,[24] in *Coelogyne stricta* and *Papilionanthe teres*, While most flavonoid classes: flavones C-glycosides, flavones, 6-hydroxyflavones, proanthocyanidins and xanthones are all represented in leaves of the Orchidaceae. Flavone C-glycosides are most common in the tropical and subtropical species belongs to the *Epidendroid* and *Vandoid* subfamilies whereas flavonol glycosides are found in *Neottioid* orchids [25]. The ethanolic extract of *Coelogyne nervosa* showed the maximum zone of inhibition against the bacteria *Pseudomonas aeruginosa* (15 mm), *Enterococcus facealis* (14.3 mm), *Salmonella enteria* (12 mm), *Bacillus subtilis* (11 mm) and

Corynebacteria spp. (9 mm) which is similar to the findings of Uma Devi *et al*, [26] and Shanmugavalli *et al*, [22] in *D. nobile, Bletilla striata* and in *Vanilla planifolia*.

The plants have abundant source of phytochemicals having important properties such as antioxidant activity. Hence, plants are being examined closely for new antioxidants, owing to the beneficial health effects of phytochemical and antioxidants [27]. Lipid peroxidation has been studied for many years. It is a primary event produced by oxidative stress or as a consequence of tissue damage, which can exacerbate tissue injury, due to the potential cytotoxicity and genotoxicity of the end products of lipid peroxidation[28]. Antioxidant reacts with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517nm and convert it to 1,1,-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate[29]. The degree of discoloration indicates the scavenging potentials of the antioxidant. Whereas, FRAP assay is based on the reduction of a ferric analogue. The Fe+++ complex of tripyridyl-triazine Fe (TPTZ)+++ gets converted into intense blue colour Fe(TPTZ)++ in the presence of any antioxidant at acidic pH[30]. The antioxidant assay determined by DPPH radical scavenging activity shows that the aqueous extract of *Coelogyne nervosa* leaves with IC_{50} value 126µg/ml and the ferrous reducing ability of various extracts of *Coelogyne nervosa* leaves was in the range of 130.59–499.65µM of AAE/g Dry weight, this shows that the plant have high antioxidant activity. Several compounds like 9,10-Dihydro-5Hphenanthro-(4,5 bcd)-pyrans and pyrones were isolated from a number of species like *Coelogyne*, *Pholidota*, and *Otochilus* [31] whereas, trans-3,4,30,50-tetrahydroxystilbene is reported to possess a chemopreventative agent with anti-leukemic activity and is being extensively studied for various cancers including colorectal and lung cancer as well as in cardiovascular diseases[32]. The anticancer activity results states that the IC_{50} value of water extract on MCF-7 cell line was 292.8µg/ml which shows the plant have anticancer activity.

Hence orchids were commonly used as traditional medicine, many of the medicinal orchids were yet to be explored for their medicinal properties, therefore in the present study indicated that *Coelogyne nervosa* possess a good antibacterial, antioxidant and anticancer 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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