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IN-VITRO ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *Cipadeessa baccifera* (ROTH) MIQ. AND *Elytraria acaulis* (L.F) LINDAU

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ABSTRACT The present study reveals the antibacterial activity and phytochemical analysis of *Cipadessa baccifera* and *Elytraria acaulis* which was carried out. Antibacterial activity determined by disc and agar well diffusion methods and 'Minimal Inhibitory Concentration' (MIC) and 'Minimal Bactericidal Concentration' (MBC) of the plant extract were determined. The plant material was powdered and successively extracted with different solvents based on their polarity. The petroleum ether, toluene, chloroform, methanol and ethanol extract of *Cipadessa baccifera* and *Elytraria acaulis* were investigated for antibacterial activity. Standard phytochemical screening tests for alkaloids, carbohydrates, phenolic compounds, steroids, tannins, phlobatannins, tannins, terpenoids, saponins, flavonoids, quinones, glycosides and amino acid were also conducted. Among the different extracts tested, methanolic extract of *Cipadessa baccifera* and *Elytraria acaulis* showed potential *in vitro* bioactivities and phytochemical constituents. The present research will be carried out to find biologically active compounds that may serve as a lead in development of new pharmaceuticals.

KEYWORDS : Antibacterial activity, phytochemicals, Cipadessa baccifera, Elytraria acaulis.

Introduction

Medicinal plants have been used as a traditional treatment for numerous human diseases for thousands of years and in many parts of the world. Hence, researchers have recently paid attention to safer phyto-medicines and biologically active compounds isolated from plant species used in herbal medicines with acceptable therapeutic index for the development of novel drugs. (Pavithra, 2010). These plants are making backbone of traditional medicinal systems in India (Nayak, 2011). The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics (C.M.Kunin, 1993). Due to increased resistance of many microorganisms towards established antibiotics, investigation of the chemical compounds within traditional plants has become desirable (Great Yarmouth, 1991).

Cipadessa baccifera belongs to Meliaceae family which is an evergreen shrub growing up to a height of 3 meters. It is very good for treatment for snake poison and particularly for the Cobra poison. Treatment includes drinking one ounce of leaf juice and applying paste of leaves in the case of cobra bite. Daily intake of one or two leaves will make a permanent resistance against cobra poison. It is also very good for Hemiplegia (condition in which one-half of a patient's body is paralyzed). It also will dilute the clotted Blood. Intake of half ounce of leaf juice every morning will help Hemiplegia patients to get cured.

Elytraria acaulis belongs to family Acanthaceae, which is a small shrub that grows in shady dry places. *Elytraria acaulis*is is widely distributed in South Africa and India. *Elytraria acaulis*is traditionally used in the treatment of asthma, migraine, leucorrhoea, snake bite etc. The *Elytraria acaulis* extracts are effective in decreasing blood glucose level, increases oral glucose tolerance test, moderately alternating body weight and there is a marked reduction in the liver glycogen levels and reduction in glycated haemoglobin levels. (Praveen Kumar R, 2014)

Materials and Methods

Plant Material

Mature and healthy plants of *Elytraria acaulis*(L.f) Lindau belonging to the family Acanthaceae was collected from Thiruvannamalai district of Tamil Nadu, India. The mature and healthy leaves of *Cipadesssa baccifera* collected from Jnanabharathi Campus, Bangalore University, Bangalore. The plants were identified by Prof. Seetharam from Biological Science Department, Bangalore University, Bangalore.

Test Organisms

The test organisms used for this study were namely: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi.* All the clinical isolates were checked for purity and maintained on Nutrient agar at 40^0c in the refrigerator until required for use.

Standardization of the Bacterial Cell Suspension

A loopful of culture was inoculated to 5ml of sterilised nutrient broth and kept the tubes for incubation for 3-4 hrs. Turbidity of the organism was observed after the incubation period and this broth culture was used to determine the efficacy of the antibacterial activity.

Preparation of aqueous extract

Fifty grams of each plant material was macerated separately with 250 ml of sterile distilled water and centrifuged at 4000 g for 30 min. The supernatant was filtered through WhatmannNo. 1 filter paper and concentrated separately under reduced pressure using rotary flash evaporator. After complete evaporation of the water, each of these extracts was kept in air-tight containers at 4^0C until tested. The dried crude plant extracts were re-suspended in Dimethylsulphoxide (DMSO) to a final concentration of 100 mg/ml and filtered through 0.45µm membrane filter for sterilization and subjected to antibacterial activity assay at 2 mg/disc (Thippeswamy *et al.,*2012).

Preparation of Solvent extract

Leaves of *Cipadessa baccifera* and *Elytraria acaulis* was collected and washed thoroughly, shade dried, pulverized mechanically and sieved. 50g of each shade dried and finely powdered of *Cipadessa bacciferra* and *Elytraria acaulis* were filled in the thimbleseparately and extracted successively with 200-300 ml of petroleum ether, toluene, chloroform, methanol and ethanol using a soxhlet extractor until colourless extract obtained on the top of the extractor. Each of the solvents extracts were concentrated separately under reduced pressure using rotary flash evaporator (Mohana *et al.,* 2009). The concentratedextracts were subsequently dried at room temperature under a steam of cold air and kept in air-tight containers at 4^0C until tested. The dried organic plant extracts were re-suspended inDMSO for the final concentration of 20 mg/ml and filtered through 0.45µm membrane filter for sterilization and subjected to evaluation of antibacterial activity by disc diffusion and agar well diffusion methods against test organisms like *Staphylococccus aureus, E.coli, Pseudomonas aeruginosa, Salmonella typhi* After the incubation period, 20mg/ml of Petroleum ether extract, toluene and methanolic extract of *Cipadessa baccifera* and petroleum ether and methanolic extract of *Elytraria acaulis* showed the maximum zone of inhibition compared to other solvents.

Further the concentration was increased as 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml for the solvents which showed the maximum inhibition for further analysis.

Determination of antibacterial assay by disc diffusion method

The 20 ml of sterilized Mueller Hinton Agar was poured into sterile petriplates after solidification, 100 µl of fresh culture of human pathogens were swabbed on the respective plates. The discs were kept over the agar plates using sterile forceps at various concentrations from 20mg/ml through 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml. The plates were incubated for 24 hours at 37^0C. After incubation, the diameter of inhibitory zones formed around each discs were measure in millimetre (mm). The readings were taken in three different fixed directions in all 3 replicates and the average value was tabulated.

Determination of antibacterial assay by agar well diffusion method

The 20 ml of sterilized Mueller Hinton Agar (MHA) was poured into sterile petriplates, after solidification, 100µl of fresh culture of human pathogens were swabbed on the respective plates. The wells were punched over the agar plates using sterile 5mm cork borer at various concentrations from 20mg/ml through 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml of which each plant extract were added to the wells. The plates were incubated for 24 hours at 37^0C. (Mahalingam, 2011) After incubation the diameter of inhibitory zones formed around each wells were measured in mm. The readings were taken in three different fixed directions in all 3 replicates and the average value was tabulated (Preethi R, 2010).

Determination of antibacterial susceptibility test using Hexa discs

Sterilized MHA was poured into the sterilized petriplates, after solidification the inoculum of *Staphylococcus aureus, E.coli, Pseudomonas aeruginosa* and *Salmonella typhi* were swabbed on the media plates. Then carefully placed the commercially available hexa discs (purchased from HiMedia) in the centres of the media with the help of foreceps and gently pressed the disc. Incubated the plates at 37^0 C for 24 hrs. After the incubation, measured the zone of inhibition and compared with samples. The disc was devoid of extracts but having the same amount of DMSO which served as negative control. The standard antibiotics such as Cephotaxime, Augmentin, Erythromycin, Chloramphenicol, Ofloxacin and Co-Trimoxazole were used as positive control.

Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

A standard two fold broth dilution technique was employed for determination of MIC and MBC. 20 mg /ml were initially dissolved with 100µl of DMSO and incorporated separately into sterile test tubes containing 10 ml of Mueller Hinton Broth (MHB) to obtain a stock concentration. Then serial two fold dilutions were made in concentrations ranged from 20 mg/ml to 0.0197 mg/ml. A 100µl of standardized each bacterial suspension was inoculated separately into the extract impregnated MHB. The MHB which was devoid of extract and the presence of the same amount of DMSO served as a negative control. All the tubes were incubated at 37^0C for 24 hrs. After incubation 50µl of broth was taken from the tubes and re-inoculated on extract free MHA plates and incubated at 37^0C for another 24 hrs. The MIC is defined as the lowest concentration of the extract required to inhibit whereas MBC is defined as the lowest concentration of the extract required to complete inhibition of bacterial growth on extract free MHA medium. Broth dilution method allows monitoring of activity over the duration and more accurate representation of antibacterial activity.

Phytochemical analysis

Phytochemical analysis of petroleum ether, toluene, chloroform and methanol extracts was carried out for *Cipadessa baccifera* and *Elytraria acaulis* respectively.

Preliminary qualitative analysis

1. Test for Alkaloids

The reconstituted extracts were acidified by adding diluted Hydrochloric Acid (HCl) which was shaken well and filtered. The acid layer (filtrate) was taken and tested for presence of alkaloids.

(a) Dragendorff's test

Taking 2 ml of extract and adding few drops of Dragendorff's reagent formed a reddish brown coloured precipitate that indicated the presence of alkaloids.

(b) Wagner's test

Taking 2 ml of extract and adding few drops of Wagner's reagent (Iodine in Potassium iodide) formed brown coloured precipitate that indicated the presence of alkaloids.

(c) Mayer's test

Taking 2 ml of extract and adding few drops of Mayer's reagent (Potassium mercuric iodide solution) formed cream coloured precipitate that indicated the presence of alkaloids.

2. Test for Carbohydrates

(a) Fehling's test

Taking 2 ml of extract and adding 1 ml mixture of equal parts of Fehling's solution A and B were boiled for few minutes. Formation of red or brick red coloured precipitate indicated the presence of reducing sugar.

(b) Molisch's test

Taking 2 ml extract and adding 1 ml of Molisch's reagent and followed by few drops of concentrated sulphuric acid (H_2SO_4). A Red-violet ring appears between two layers that indicate the presence of carbohydrates and further disappear on the addition of excess alkali.

3. Test for Phenols

(a) 2ml of extract was treated with aqueous 5% FeCl3 and observed for formation of deep blue or black colour indicated the presence of phenols.

4. Steroids

(a) Liebermann-Burchard's test

2 mg of dry extract was dissolved in acetic anhydride and heated to boiling. Then was cooled and was added 1 ml of concentrated sulphuric acid along the sides of the test tube. Formation of green colour indicated the presence of steroids.

(b) Salkowski reaction

2 mg of dry extract was shaken with chloroform, then to the chloroform layer sulphuric acid was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids.

5. Tannins

(a) Taking2 ml of the extract and adding few drops of $FeCl_3$ solution (5% w/v). A green colour indicated the presence of gallotannins, while brown colour indicates the presence of pseudotannins.

6. Test for Phlobatannins

(a) 2ml of extract was boiled with 1 ml of aqueous HCl which gives deposition of a red precipitation which indicated the presence of phlobatannins.

7. Test for Triterpenoids

(a) Liebermann - Burchard's test

Taking 2 ml of extract and adding few drops of acetic anhydride and conc. H_2SO_4 along the sides of test tube developed deep red colour that indicates the presence of triterpenoids.

8. Test for Saponin

(a) Foam Test:

2ml of extract was mixed with water and was shaken vigorously resulting in the formation of froth which was stable for atleast 15 minutes. This indicated the presence of saponin.

9. Test for Flavonoids

(a) Taking 2 ml of extract and adding few drops of ferric chloride ($FeCl_3$) solution shows intense green colour that indicates the presence of flavonoids.

10. Test for Quinones

(a) 2ml of extract was treated with Conc. HCl and observed for the formation of yellow precipitation (or colouration).

11. Test for Glycosides

(a) Keller Killiani test

Taking 2ml extract and adding few drops of $FeCl_3$ solution followed by

few drops of conc. H_2SO_4 slowly reveals a reddish brown and upper acetic acid layer turns bluish green colour indicating the presence of glycosides.

(b) Molisch's test

Taking 2 ml extract and adding 1 ml of Molisch's reagent and followed by few drops of concentrated sulphuric acid (H_2SO_4). A Red-violet ring appears between two layers that indicate the presence of carbohydrates and further disappear on the addition of excess alkali.

12. Test for amino acids

(a) Ninhydrin test:

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of extract. Appearance of purple colour indicates the presence of amino acids.

Result:

Table No.1

Determination of antibacterial assay of *Cipadessa baccifera*

Test Organism	Zone of Inhibition in mm				
	Petroleum ether extract	Toluene extract	Chloroform extract	Methanolic extract	Ethonolic extract
S. aureus	12.3±1.247	8.3±0.472	00±0.000	7.6±0.476	8.6±0.476
E.coli	14.3±0.472	8.6±0.476	10.0±0.816	7.3±0.472	10.0±0.000
Pseudomonas aeruginosa	00.0±0.000	00.0±0.000	8.3±0.472	00±0.000	6.3±0.472
Salmonella typhi	10.6±0.476	00.0±0.000	00.0±0.00	12.3±0.472	7.3±0.472

Values are mean inhibition zone (mm) ± S.D of three replicates

Table No.2

Determination of antibacterial assay of *Elytraria acaulis*

Test Organism	Zone of Inhibition in mm				
	Petroluem ether extract	Toluene extract	Chloroform extract	Methanolic extract	Ethanolic extract
S.aureus	14.6±0.476	14.3±0.435	15±0.8164	15.6±0.476	13.6±0.476
E.coli	13.6±0.476	0.0±0.00	12.6±0.476	14.3±0.435	10.6±0.476
Pseudomonas aeruginosa	00.0±0.000	00.0±0.00	8.3±0.435	9.0±0.000	00.0±0.00
Salmonella typhi	10.3±0.435	12.6±0.476	10.0±0.816	12.3±0.435	00.0±0.00

Values are mean inhibition zone (mm) ± S.D of three replicates

Table No. 3 Determination of antibacterial assay of *Cipadessa baccifera* at different concentrations

	Petroleum ether					Toluene					Methanol				
	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml
S. aureus	13.3±0.7893	16.6±1.98	18.3±0.7893	19.3±1.5349	20.3±0.7893	17.6±1.57	19.3±1.3	22.6±1.35	22±1.414	25.3±0.7893	12.3±0.7893	13.3±0.7893	14±0.00	15±0.00	18±0.4714
E.coli	12.6±0.7893	13.3±0.7893	14.3±0.472	16.6±1.98	18.3±0.7893	8.3±0.7893	8.6±0.476	9±0.000	10.3±07893	10.±0.7893	7.3±0.472	8.6±0.476	9.3±0.7893	10±0.00	10.6±0.7893
Salmonellatyphi.	18.3±0.7893	19.6±1.3512	20±0.816	21.6±1.3512	23.3±0.7893	20±1.632	22.6±1.04	25±0.8164	25.3±1.0754	26.6±1.0456	10.3±0.435	11.6±0.476	12±0.00	12.6±0.476	13.3±0.435

Values are mean inhibition zone (mm) ± S.D of three replicates

Table No. 4 Determination of antibacterial assay of *Elytraria acaulis* at different concentrations

	Petroleum ether					Methanol				
	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml	20mg/ml	40mg/ml	60mg/ml	80mg/ml	100mg/ml
S. aureus	18.3±0.435	19.0±0.816	23.6±0.476	25.3±0.435	29.0±0.00	9.0±0.000	11.3±0.789	13.0±0.00	13.0±0.000	13.3±0.789
Salmonellatyphi.	18.3±0.435	20.6±0.476	24.3±0.435	24.0±0.00	25.6±0.476	11.0±0.816	15.6±1.351	14.3±0.7893	16.6±1.35	17.3±0.789

Values are mean inhibition zone (mm) ± S.D of three replicates

Table No.5 Determination of Antibacterial susceptibility Test using Hexa discs

			Zone of Inhibition in mm			
Antibiotics	Symbol	Concn.	*S. aureus*	*E.coli*	*P.aeruginosa*	*Salmonellatyphi*
Cephotaxime	Ce	30 mcg	30.6±2.625	23.0±2.160	19.0±0.000	15.6±2.868
Augmentin	Au	30 mcg	23.3±0.943	14.3±0.435	00.0±0.000	10.6±0.476
Erythromycin	E	10 mcg	27.6±2.868	23.0±2.160	15.6±0.476	29.6±0.476
Chloramphenicol	C	30 mcg	28.0±2.943	30.3±1.660	20.0±0.8164	25.3±1.700
Ofloxacin	Of	5 mcg	24.0±0.8164	29.3±2.055	30.0±0.8164	30.3±0.435
Co-Trimoxazole	Co	25 mcg	31.6±0.476	26.3±2.624	15.0±0.8164	16.3±0.435

Values are mean inhibition zone (mm) ± S.D of three replicates

Table No.6 Determination of MIC and MBC of *Cipadessa baccifera* and *Elytraria acaulis*

Sample	Solvent Plant extracted	Organism used	MIC mg/ml	MBC mg/ml
Cipadessabaccifera	Petroleum ether	*Staphylococcus aureus*	2.5	>5
		E.coli	0.625	10
		Pseudomonas aeruginosa	5	10
		Salmonella typhi	2.5	>5
	Toluene extract	*Staphylococcus aureus*	0.625	2.5
		E.coli	-	-
		Pseudomonas aeruginosa	1.25	10
		Salmonella typhi	-	-
	Methanol	*Staphylococcus aureus*	1.25	>5
		E.coli	-	-
		Pseudomonas aeruginosa	-	-
		Salmonella typhi	-	-
Elytrariaacaulis	Petroleum ether	*Staphylococcus aureus*	1.25	>5
		E.coli	-	-
		Pseudomonas aeruginosa	-	-
		Salmonella typhi	-	-
	Methanol	*Staphylococcus aureus*	2.5	10
		E.coli	-	-
		Pseudomonas aeruginosa	-	-
		Salmonella typhi	2.5	>5

Table No.7 Preliminary Qualitative Phytochemical Analysis of *Cipadessa baccifera* and *Elytraria acaulis*

	Cipadessa baccifera				*Elytraria acaulis*			
Phytochemicals	Petroleum ether	Toluene	Chloroform	Methanol	Petroleum ether	Toluene	Chloroform	Methanol
Alkaloids	+	+	-	+	+	-	-	+
Carbohydrates	-	-	+	+	-	-	-	+
Phenolic compounds	+		+	+	+	+	+	+
Steroids	+	-	-	+	-	-	-	+
Tannins	-	+	+	+	+	+	+	+
Phlobatannins	-	+	+	+	-	-	+	+
Terpenoids	-	-	-	+	-	-	-	+
Saponins	+	+	+		+		+	+
Flavonoids	+	-	-	+	+	-	-	-
Quinones	+	-	-	+	+	-	-	+
Glycosides	+	-	-	+	-	-	-	+
Amino acid	-	+	+	-	-	+	+	+

+ = present; - = absent

Discussion

Antibacterial activities of both the plants were investigated by disc diffusion and agar well diffusion method. As per the investigation petroleum ether, toluene and methanolic extract of *Cipadessa baccifera* and petroleum ether and methanolic extract of *Elytraria acaulis* showed the effective and potential activity against the *Staphylococcus aureus* and *Salmonella typhi* Toluene extract showed effective against *Staphylococcus aureus* and *E.coli*. Petroleum ether extract of *Elytraria acaulis* showed effective against *Staphylococcus aureus, Salmonella typhi*. The methanolic extract of *Elytraria acaulis* showed effective against *Staphylococcus aureus* and *Salmonella typhi*. Overall *staphylococcus aureus* expressed its potential antibacterial activity against all the plant extracts except chloroform extract of *Cipadessa baccifera*. Therefore, the orders of ease of susceptibility of the test organisms were *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa* and finally *E.coli.*

The standard antibiotics used for this experiment was shown in the table No. 5. According to the table *Staphylococcus aureus* showed the maximum zone of inhibition (ZOI) against Co-Trimixazole and Cephataxime, *E.coli* showed maximum ZOI against Chloramphenicol, *Pseudomonas aeruginosa* and *Salmonella typhi* showed maximum ZOI against oflaxacin.

The MIC and MBC values of petroleum ether extract of *Cipadessa baccifera* ranging from 2.5mg /ml to >5mg/ml against *Staphylococcus aureus*, 0.625 mg/ml and >10 against *E.coli*, 10mg/ml and >5 against *Pseudomonas aeruginosa*and 0.625mg/ml to>2.5mg/ml against *Salmonella typhi*. and the toluene extract of *Cipadessa baccifera* ranging from 0.625mg/ml and >2.5 mg/ml against *S. aureus* and 1.25mg/ml and >10 against *Pesudomonas aeruginosa*, methanolic extract of *Cipadessa baccifera* ranging from 1.25mg/ml and >5mg/ml against Staphylococcus *aureus* respectively. Similarly petroleum ether extract of *Elytraria acaulis* ranging from 1.25mg/ml and >5mg/ml against *Staphylococcus aureus* and methanolic extract of *Elytraria acaulis* ranging from 2.5mg/ml and >10mg/ml against *Staphylococcus aureus* and 2.5mg/ml and >5mg/ml against *Salmonella typhi* respectively. However in determining the MBC, some of the extracts with good MIC values did not have a corresponding good MBC suggesting such extracts have strong bacterio static activity than bactericidal activity. Petroleum ether extract of *Cipadessa baccifera* showed lowest MIC value ranging from 0.625mg/ml and >10mg/ml. Further investigations such as isolation and characterization of the active compounds responsible for antifungal activity and toxicological experiments using animal models are required before clinical application.

The petroleum ether, chloroform, Toluene and methanol extracts of both the plants were subjected to qualitative preliminary phytochemical analysis with different chemical reagents. The results are tabulated (table No. 7). These secondary metabolites contribute significantly towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic, antimalarial, anticholinergic, anti-leprosy activities etc (Negi, 2011). From the results, it was found that our plant species contains many effective compounds like alkaloids, carbohydrates, phenolic compounds, steroids, tannins, phlobatannins, tannins, terpenoids, saponins, flavonoids, quinones, glycosides and amino acid.

Data given are the mean of four replicates ± S.D error ($P \leq 0.05$)

Statistical analysis

Results were expressed as mean value ± standard error of the mean (SEM) of growth inhibition zones diameters obtained with those natural products which amount was sufficient to perform repetitions.

Conclusion

The present research work revealed that the extracts of petroleum ether, toluene and methanolic extract of *Cipadessa baccifera* and petroleum ether and methanolic extract of *Elytraria acaulis* exert appreciable antibacterial effect on all the test organisms and chloroform and ethanolic extract of *Cipadessa baccifera* and except petroleum ether and methanolic extract of *Elytraria acaulis* showed least effective on all the test organisms. Furthermore, this study also revealed that the petroleum ether, toluene and the methanolic extract of both the plants had the highest antibacterial effect on all the test organisms, therefore *Cipadessa baccifera* and *Elytraria acaulis* are recommended for the usage by the local populace because of its significant antibacterial effect as revealed. These secondary metabolites contribute significantly towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, anti-inflammatory activities etc. Further the secondary metabolites were identified as suitable for future research analysis like TLC, HPLC, and GCMS etc.

Cipadessa baccifera (Roth)Miq.

Elytraria acaulis (l.f)Lindau

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