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RESEARCH ARTICLE

Phytochemical Screening, Antioxidant, Antimicrobial and Quantitative Multi-Elemental Analysis of *Habenaria longicorniculata* J. Graham

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Abstract

Western Ghats of India are known to be a major biological hotspot that supports the plant diversity and endemism. Orchidaceae are among the most evolutionarily and ecologically significant plants known for a wide variety of epiphytic and terrestrial growth forms and hardiness; they successfully colonize almost every habitat on earth, including soil (terrestrial), rock surfaces (lithophytic) and other plants (epiphytic). *Habenaria longicorniculata* J. Graham representative species of orchidaceae is famous for its use as medicinal herbs. The phytochemical study, quantitative multi-mineral analysis by Inductively Coupled Plasma (ICP) spectrometry, antioxidant and antimicrobial activities of the tuber extracts of *Habenaria longicorniculata* J. Graham were evaluated. Phytochemical screening indicated that, plants are rich in a variety of primary and secondary metabolites such as carbohydrates, glycosides, alkaloids, vitamin C, vitamin E, flavonoids, phenols and saponins. A high-throughput micro-scaled method has been developed which enables digestion of small quantities of plant samples for subsequent elemental profiling by ICP-spectrometry. This piece of work highlights the biochemical and ethnopharmacological significance of *H. longicorniculata.*

Keywords: Western Ghats, *Habenaria longicorniculata*, phytochemicals, antioxidant, multi-mineral analysis.

Introduction

Orchidaceae, which comprises an estimated 20000 to 35000 species, constitutes one of the most diverse families (Dressler, 1993) and most species of this family have medicinal and ornamental properties (Singh, 2001). Orchidaceae are among the most evolutionarily and ecologically significant plants known for a wide variety of epiphytic and terrestrial growth forms and hardiness; they successfully colonize almost every habitat on earth, including soil (terrestrial), rock surfaces (lithophytic) and other plants (epiphytic). *Habenaria longicorniculata,* long-tailed is a 1-3 ft high terrestrial herb, oblong-elliptic leaves are 3-10, clustered on the base and lie flat on the ground. Flowers occur on a long erect stalk which is 30-80 cm tall. White fragrant flowers are 1-4 in number. Greenish white petals are 10-14 mm long. Flowers have a spur which is 10-15 cm long. Overall, the flowers have the shape of an inverted funnel. It was first collected by John Graham from Khandala (Graham, 1839).

Genus Habenaria Wild belongs to large genus family Orchidaceae with 83 species from India (Mishra, 2001). Orchid phytochemicals are generally categorized as alkaloids, flavonoids, carotenoids, anthocyanins and sterols. Among orchids, Dendrobium is the leading genus for phytochemical content and Zhang *et al*. (2003) reviewed 100 compounds from 42 Dendrobium species, including 32 alkaloids, 6 coumarins, 15 bibenzyls, 4 fluorenones, 22 phenanthrenes and 7 sesquiterpenoids. Williams (1979) conducted a major survey of leaf flavonoids and surveyed 142 species in 75 genera and found that the most common constituents were flavones C-glycoside and flavonols. To date, more than 2000 orchid species have been screened for their alkaloids and/or flavonoids contents. Altogether, a single familial pattern of flavonoid distribution is not evident in orchids. The root decoction of *Habenaria commelinifolia* administered orally on an empty stomach to cure spermatorrhoea and urinary trouble has been reported (Kirtikar and Basu, 1981; Singh, 2001; Jalal, 2008). The tuber paste of *Habenaria furcifera* (Lindl.) is used as an ointment for cuts, wounds and poisonous bites (Roy, 2007). The whole fresh plant paste of *Habenaria hollandiana* Sant. is applied externally for scorpion sting and also for maggot infested sores (Roy, 2007). The tuber paste of *Habenaria longicorniculata* (Graham) with turmeric is used as an external application for leucoderma (Roy, 2007). A tuber decoction of *Habenaria marginata* (Coleb) is taken orally to treat malignant ulcer (Leander, 1967; Hossain, 2009). The leaves and tubers of *Habenaria pectinata* (D. Don) are crushed and applied as an antidote to snake bites. Tubers mixed with condiments are used in arthritis (Chauhan, 1990; Singh and Duggal, 2009). *Habeneria roxburghii* (Nicolson) tuber decoction is applied externally for snake bite (Roy, 2007).

In the market, there is no clear cut distinction between Riddhi and Vriddhi. The larger tubers of various species of Habenaria are used as Vriddhi and the smaller ones as Riddhi. Ayurvedic system of medicine, a rejuvenating herbal formulation 'Astavarga' is derived from a group of 8 herbs, some of these herbs i.e. jivak (*Microstylis wallichii*), kakoli (*Habenaria acuminata*), riddhi (*H. intermedia*) and vriddhi (*H. edgeworthii*) are orchids (Handa, 1986; Singh and Duggal, 2009). *Flickingeria macraei* is used in 'Ayurveda' in the name of 'jeevanti' which is used as astringent to the bowels, aphrodisiac and in asthma and bronchitis (Kirtiker and Basu, 1975). Other commonly used orchid drugs in the Ayurvedic system are Salem (*Orchis latifolia* and *Eulophia latifolia*), jewanti (*Dendrobium alpestre*), shwethuli and rasna (*Acampe papillosa* and *Vanda tessellata*). In 'Sushrutasamhita' it is mentioned that the underground tuber of *Orchis latifolia* is used in the drug 'munjatak' which pacifies cough (Khasim and Rao, 1999). The leaves of *Vanda roxburghii* have been prescribed in the ancient Sanskrit literature for external application in rheumatism, ear infections, fractures and diseases of nervous system. Incredible diversity, high alkaloids and glycosides content, research on orchids is full of potential. The residents of India are acquainted with a far larger number of orchid species than the native of any other country in the face of the earth (Kirtikar and Basu, 1981). Several novel compounds and drugs, both in phytochemical and pharmacological point of view have been reported from orchids. Linking of the indigenous knowledge to the modern research methodology will provide impetus to discover new drugs much more effective than contemporary synthetic medicines. The present study reviews the traditional therapeutic uses of orchids and recent advances in pharmacological investigations. In the present study, we report a high-throughput micro-scaled method which enables digestion of small quantities of plant samples for subsequent elemental profiling by ICP-spectrometry, DPPH anti-scavenging activity, antimicrobial activity. The investigations led to the identification of phytochemical contents of orchids of indigenous origin.

Materials and methods

Chemicals and instruments: All solvents were distilled prior to use. TLC was performed on silica gel 60 F254 (Merck). All reagents and solvents purchased from Merck Chemicals and Himedia. UV-Vis spectra were recorded on a Shimadzu UV-1700 spectrophotometer. The HPLC were recorded on Agilent Technologies 1200 series Quaternary. Minerals detection was performed by using CEM Mars 6 microwave digester and Teledyne Leeman, ICP OES model Prodigy Dual View (Induction Coupled Plasma).

Sampling: Fresh samples of tubers of *Habenaria longicorniculata* were collected from Lonavala, Dist., Pune region of Western Ghats of Maharashtra (Fig. 1 and 2). These plants were identified and authenticated using herbarium collection at Dept. of Botany, DST-FIST School of Life Science, SRTM University, Nanded (MS), India and The plant list (2013). Fresh tubers were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. The shade dried material was ground into coarse powder using mechanical grinder (Panasonic). This coarse powder was sieved by 1 mm pore size sieve. The powder was stored in airtight containers at room temperature till further phytochemical screening of secondary metabolites.

Fig. 1. Habit of *Habenaria longicorniculata.*

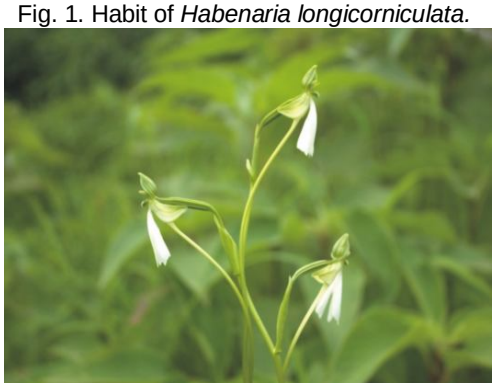
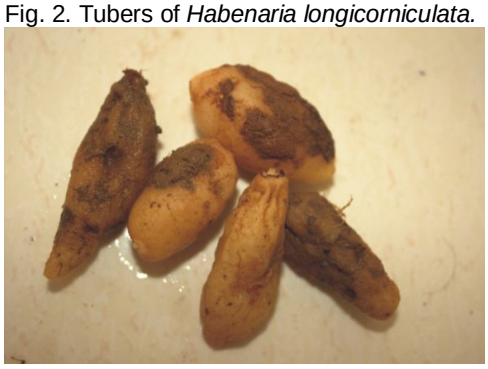


Fig. 2. Tubers of *Habenaria longicorniculata.*



Soxhlet extraction: Exhaustive Soxhlet extraction was performed using a classical Soxhlet apparatus with accurately weighed 10 g of the crude powder of plant material for 18-40 h. The solvents like water, methanol, chloroform, acetone and IPA were used for extraction. The extraction was conducted for 6-8 h/d and finally all the extracts were evaporated under vacuum. The water, methanol, chloroform, acetone and IPA extracts of tubers of the plant were prepared according to standard methods (Harbone, 1998). Nitrogen gas was purged through these extracts to prevent oxidation of secondary metabolites. These extracts were sealed in airtight containers and stored at -4°C.

Phytochemical screening: Phytochemical screening of active plant extracts was done by following the standard method of Khandelwal (2000), for the qualitative analysis of various phytochemicals such as alkaloids, carbohydrate, glycosides, saponins, flavonoids and phenols which could be responsible for antioxidant activity.

Mineral analysis

Micro-scaled digestion: CEM-MARS 6 microwave oven was used for micro-scaled digestion. About 0.5 g of herbal samples were weighed and transferred to CEM-Xpress vessels, 8-10 mL of conc. HNO_3 was added to the samples. The samples were pre-digested for 10-15 min prior to capping the vessels. The CEM-Xpress vessels were assembled for microwave irradiation. The microwave program was adjusted with respect to the number of vessels and reference to the guidelines of CEM at 1000 W with 100% level, 25 min ramping period was used to reach the digestion temperature of 180°C which there upon was maintained for 15 min. The CEM-Xpress vessels were kept in fume hood for cooling and to release the pressure by uncapping. The contents were transferred to 50 mL volumetric flasks and volume was made with distilled water. The solutions were filtered prior to use. For calibration, Leeman and Thomas Baker standard sample were used as the reference for the calibration range. The spray chamber, nebulizer and torch assembly was completely cleaned to eliminate contamination. The plasma was stabilized for 15 min by flushing with distilled water. An instrument calibration was performed to check the wavelength shift and the same was successful with a minimum deviation of <10% with master scan. After calibration, the instrument was optimized with 10 PPM solution containing elements (Table 1) and the same as optimized with maximum intensity and best BEC at the parameters mentioned in operating condition above. Diluted samples were used for further analysis by using Teledyne Leeman, ICP (Induction Coupled Plasma).

Table 1. Instrumental characteristics and setting for ICP-OES: Spectrometer Leeman Lab's simultaneous ICP-OES Prodigy XP dual system.

	Parameters range		Actual parameters
	Min	Max	
Power	0.1	2.0	1.1 Kw
Coolant flow	5	20	18 L/Min
Auxiliary flow	0.0	2.0	0.2 L/M
Nebulizer flow	5	60	34 psi
Plasma torch	-	-	Dual
Spray chamber	-	-	Cyclonic
Nebulizer	-	-	Concentric
Sample aspiration rate	0.5	2.0	1.4 mL/min
Replicate read time	-	-	40 sec per replicate for axial

Determination of vitamin E by HPLC

Standard preparation: dl α-tocopherol acetate (96%), (Vitamin E) manufactured by Merck was used for calibration of standard curves. About 1 mg of dl α-tocopherol acetate was dissolved in 1 mL in HPLC grade methanol. Dilutions of 100, 50, 25 and 10 µg/mL was prepared and the pre-treated sample extracts and stock solutions were filtered through 0.45 µm syringe filters.

Reverse phase HPLC method: The concentration of α-tocopherol (Vitamin E) in the extracts was determined by Agilent Technologies 1200 series Quaternary system, equipped with auto sampler, quaternary pump, degasser, column oven and a DAD detector. The spectral data was collected at UV detection at 220 nm. The solvent system of acetonitrile and water (95:5) was used a gradient mobile phase on Agilent ZORBAX 300 SB column (4.6 × 150 mm × 5 µm) at a flow rate of 1.0 mL/min, 10 µL injection volume and detection was optimized at 220 nm with 15 min separation time.

Vitamin C: About 0.25% ethanolic solution of DCPI (2,6-dichlorophenol-indophenol sodium salt) was prepared for the detection of Vitamin C. To 0.5 mL of sample extracts, 2 drops of DCPI indicator was added. The blue coloration changed to red confirmed the presence of vitamin C. The test was carried out for all the extracts.

Anti-scavenging activity: DPPH solution (0.1 mM) was prepared in methanol by dissolving 0.0394 g DPPH in 1000 mL methanol. The solution was kept in darkness for 30 min to complete the reaction. The free radicals scavenging activity of the crude extracts was determined by the 1,1-diphenyl-2-picryl-hydrazil (DPPH). Antioxidant activity was measured by the standard method described by Brand-Willium *et al.* (1995) wherein, the bleaching rate of stable free radical DPPH was monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbed at 570 nm, but upon reduction by an antioxidant or radical species its absorption decreased. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $(ABS_{control}-ABS_{sample})/(ABS_{control})$ X 100), whereas $ABS_{control}$ is absorbance of negative control and ABS_{sample} is the absorbance of the reaction mixture containing the sample extract.

In vitro antimicrobial activity: Plates were prepared for the assay by using standard pour plate technique for bacterial cultures. About 0.1 mL of bacterial suspension was spread onto the Nutrient Agar medium. Clinically isolated microbial strains *Escherichia coli, Pseudomonas aeuroginosa, Salmonella typhi, Staphylococcus aureus and Aspergillus niger* were used. A spore suspension of *Aspergillus* was prepared in sterile distilled water which was added with Tween-20 as a surfactant. About 0.1 mL of spore suspension was spread onto Potato dextrose Agar medium. These plates were further used for the assay purpose immediately. Paper discs approx. 6 mm in diameter were carefully cut from multiple layers of thin filter paper with a sharp cork borer. Sterilization of the discs was done by autoclaving them at 121°C for 15 min and allowed to dry before use. Hot-air sterilization reduces the absorptive capacity of the paper, but drying for 1 h at 100°C had little effect.

Table 2. Preliminary phytochemical screening of tuber extracts of *H. longicorniculata.*

Constituents	Test	Observation	Inference				
			S1	S2	S3	S4	S5
Carbohydrates	Benedict's reagent	Red precipitate	+	+	+	+	+
Alkaloids	Mayer's reagent	White precipitate	-	+	+	+	-
Glycosides	Borntranger's reagent	Pink coloration	+	+	+	+	+
Saponins	Foaming	Frothing persisted for 10-15 min	+	+	-	-	+
Flavonoids	Shinoda	Pink-Red coloration	-	+	-	-	-
Phenols	Ferric chloride	Dark brown coloration	-	+	+	+	+
Vitamin C	2,6-dichlorophenol-indophenol sodium salt	Red coloration	+	-	-	-	-
Vitamin E	HPLC method	-	-	+	+	-	-

S1=Water, S2=Methanol, S3=Acetone, S4=Chloroform, S5=Isopropyl acetate.

Paper disc was grasped with finely pointed forceps and its lower edge was touched to the surface of the agar plate. Fixed amount (10 µL) of test solution and standards were used to transfer onto the paper disk by Micropipette. Clean and Sterile forceps were used for each respective solution or the forceps were flamed and cooled between the changes of samples. The discs were kept on the agar to allow adequate room for the development of the zones of inhibition. Two discs were introduced on 90 X 15 mm petri dishes. Reference marks on the outside of the petri dishes were given for identifying the discs. The plates were incubated at 37°C for 24 h. Results were recorded in the terms of zones of inhibition in mm. The fungal culture plate was incubated at 28°C for 6 d.

Flavoinoids analysis by HPTLC: The standards Quercetin, Kaemferol, Catechin gallate, Rutin hydrate and Hesperdin were procured from Sigma Aldrich USA. All the standard solutions were prepared in ethanol where as hesperdin in water. Chromatography was performed on silica gel $60F_{254}$ (10 cm X 10 cm; 25 mm layer thickness; Merk) with aqueous, methanolic, chloroform and acetone extracts of *H. longicorniculata* tuber. The fraction residues were collected and (10 µL) subjected for HPTLC (CAMAG, Switzerland) analysis. The fractions were impregnated on silica gel $60F_{254}$ TLC plate. The plate was air-dried and then inserted in CAMAG-twin through lass chamber containing solvent system of composition with ethyl acetate, acetic acid, formic acid and water (100:11:11:27) as a gradient mobile phase for 20 min. The well eluted TLC plate was then dried at 105°C for 15 min and scanned using Scanner 3 (CAMAG, Switzerland) at 254 and 366 nm using Win Cat 4 software.

Results and discussion

Optimization of extraction method: In order to extract the phytochemicals from herbal samples efficiently, variables involved in this procedure were optimized, including extraction solvent (Water, Methanol, Chloroform, Acetone, IPA, 100%), extraction method (Soxhlet, reflux, percolation) and extraction time (18-40 h). The extraction time in water was 40 h. The biomass was refluxed for 40 h and dried naturally for 2-3 d.

To the dried biomass, 100% methanol was added and the reaction was percolated to extract phytochemicals. The methanolic fraction was collected in amber colored bottle under nitrogen atmosphere. The material was dried for 5-6 h. The procedure was repeated for chloroform, acetone and IPA. The extraction time was optimized for all the samples. All the extracts were preserved under nitrogen atmosphere in amber colored bottles at -4°C.

Phytochemical screening: It is known that plants are rich in a variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, phenols, steroids, glycosides, saponins and volatile oils. It is necessary to identify the phytochemical components of local medicinal plants usually employed by herbalists in the treatment of diseases (Banso and Adeyemo, 2007). The presence or absence of certain phytochemicals could be used to explain some of the biological activity of certain plant extracts. For example, saponins are a special class of glycosides which have soapy characteristics and have been reported to be active antifungal agents. Antimicrobial properties of a number of tannins, flavonoids, alkaloids have been reported. Not only the antimicrobial properties have been ascribed to these plant phytochemicals, but other biological activities including modulation of the immune system have been assigned to these compounds in plants. Phytochemical screening of the tuber extracts of *Habenaria longicorniculata* revealed the presence of different phytochemicals. Indeed, phytochemical investigations of the plants *H. longicorniculata* have resulted in occurrences of carbohydrates, alkaloids, glycosides, saponins, flavanoids, phenols, vitamin E and vitamin C. Table 2 illustrates the results of phytochemical screening of all the extracts of *H. longicorniculata*. The qualitative analysis of carbohydrates (Benedict's reagent test) and glycosides (Borntranger's reagent) were carried out in all extracts i.e. aqueous (S1), methanol (S2), acetone (S3), chloroform (S4) and isopropyl acetate (S5) extracts of *H. longicorniculata*. The solutions turned red and pink confirmed the presence of carbohydrates and glycosides respectively. The hydrophilic carbohydrates and glycosides were present in water (S1) whereas and hydrophobic carbohydrates and glycosides were detected in rest of the organic solvents (S2-S5).

Table 3. Accuracy of elemental concentrations in *H. longicorniculata* after micro-scaled digestion.

Elements (ppm)	*H.longicorniculata*
Zn	12.673
Cu	15.9086
Mn	125.1686
Se	0
Fe	4805.79

The Mayer's test of extracts S2, S3, S4 displayed appearance of white turbidity for alkaloids. The alkaloids were absent in S1, S5 extracts of *H. longicorniculata.* The dark brown coloration test for phenols was observed in S2-S5. No traces of phenols are found in water. The extracts S1-S5 were shaken with distilled water. The persistence of froth in S1, S2 and S5 was observed, indicated the presence of saponins. The hydrophilic flavonoids were detected in extract S1. The water soluble vitamin C was found in *H. longicorniculata.* Vitamin E was qualitatively and quantitatively analyzed by HPLC method in tuber extracts S2, S3, S4 of *H. longicorniculata*.

Optimization and calibration of H. longicorniculata tuber extracts: Iron and copper are of great importance for life. As redox-active metal, they are involved in photosynthesis, mitochondrial respiration, nitrogen assimilation and hormone biosynthesis. Manganese is essential for plant metabolism and development and occurs in oxidation states II, III, and IV in approximately 35 enzymes of a plant cell. Zinc is important as a component of enzymes for protein synthesis and energy production and maintains the structural integrity of bio-membranes. Most of the zinc enzymes are involved in regulation of DNA-transcription, RNA-processing and translation. Although the essentiality of Se to plants has not been established yet, Se is considered as a beneficial element in promoting plant growth in some plant species. The focal point of our study was to develop effective digestion method for the preparation of mineral analysis by ICP. Microwave digestion is a common technique used by elemental scientists to dissolve heavy metals in the presence of organic molecules prior to analysis by inductively coupled plasma, atomic absorption, or atomic emission measurements (Kingston and Lois, 1988). Quantitative multi-elemental analysis by inductively coupled plasma (ICP) spectrometry depends on complete digestion of solid samples. However, fast and thorough sample digestion is a challenging analytical task which constitutes a bottleneck in modern multi-elemental analysis. Additional obstacles may be that sample quantities are limited with low elemental concentrations. In such cases, digestion in small volumes with minimum dilution and contamination is required in order to obtain high accuracy data. After optimization, a new calibration method was created for measuring these samples; the wavelengths used for calibration were Cu 324.754 nm, Mn 257.610 nm, Se 196.090 nm, Fe 259.940 nm and Zn 213.856 nm.

Calibrated STD solutions were measured 3 times one by one with an RSD <1%. Once all the calibration standards are finished, a necessary background correction was applied for each wavelength. The results are depicted in Table 3. We have developed a micro-scaled microwave digestion procedure and optimized it for accurate elemental profiling of plant materials. A commercially available 40-position rotor with 5 mL Polytetraflouro ethylene (PTFE) vials, originally designed for microwave-based parallel organic synthesis were used as a platform for the digestion. The novel micro-scaled method was successfully validated by the use of various certified reference materials (CRM). The micro-scaled digestion procedure was applied on crude powder of dried plant material in small batches. We have determined 5 elements in aqueous extract given in Table 3. Thereby, the concentration of minerals in plant extracts had different profiles and quantitative differences had been detected. The most abundant microelement was Fe in *Habenaria longicorniculata* whereas, copper was found at the lowest concentration. The content of Fe was especially high in comparison to Zn, Cu, Mn and in consequence, Se was not detected. The concentration of Zn content was less abundant. Vitamin E, C, carotenoids, Se and other trace minerals are important antioxidant components of animal diets and their roles in animal health and immune function are indispensable. In addition, several metallo- enzymes which include glutathione peroxidase (Se), catalase (Fe) and superoxide dismutase (Cu, Zn and Mn) are also critical in protecting the internal cellular constituents from oxidative damage. Only when these metals are delivered in the diet in sufficient amounts can the animal body synthesize these antioxidant enzymes. In contrast, deficiency of those elements causes oxidative stress and damage to biological molecules and membranes (McDowell, 2007). Orchid contains macro and microelements and can be used as a supplementary food for the vulnerable groups including children and old people (Makarius *et al.,* 2013).

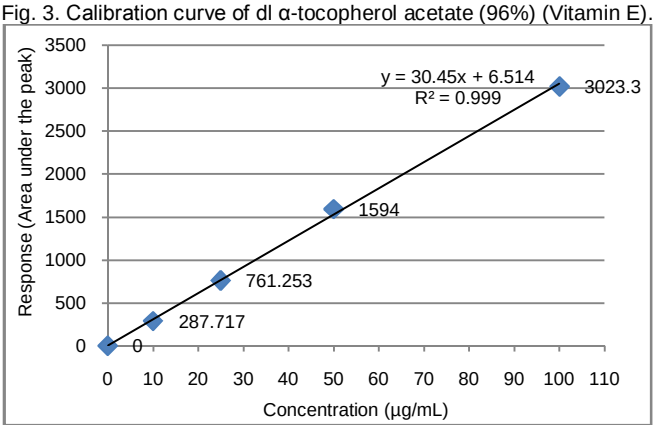
Determination of Vitamin E by HPLC: Vitamins are a diverse group of organic compounds essential in trace amounts for the normal growth and maintenance of life. To ensure the adequate intake of vitamins, the human diet can be completed with a high range of multivitamin tablets and food products enriched with vitamins, in other words, these compounds are usually administered as nutraceutical or functional ingredient. They are classified as either water-soluble or fat soluble. Vitamin E is fat-soluble whereas Vitamin C is water-soluble. Vitamin E is a generic term for tocopherols and tocotrienols and it is fat-soluble antioxidant that blocks the production of reactive oxygen species formed when lipids undergoes oxidation. We employed reverse phase HPLC-analytical tool for qualitative estimation of vitamin E, in which HPLC has been coupled with UV detector.

Optimization of HPLC method: To meet the requirements for quantitative analysis, various HPLC parameters were

examined, including different columns (Agilent SB-C18 length 250 mm and 150 mm, width 4.6, particle size 5 µm), column temperature (25°C) and UV wavelength (220 nm). The best chromatographic resolution was obtained on Agilent SB-C18 length 4.6 X 150 mm, 5 µm column at 25°C. The UV detector was monitored at 200-380 nm for fingerprinting analysis because the peaks were observed under this wavelength. The high intense peak was observed at 220 nm.

Method validation and calibration: A calibration curve is simply a graph where concentration is plotted along the x-axis and area is plotted along the y-axis (Response, absorbance, intensity, peak height, etc.). The line represents the calibration curve. Figure 3 showed a calibration curve of vitamin E. We have constructed a calibration curve for vitamin E. It was created by running 4 different calibration standards (10, 25, 50 and 100 µg/mL). Each concentration gave a peak area (287.717, 761.253, 1594, 3023.3) respectively. Peak area was then plotted against the concentrations. The linear trend line has been drawn and linear regression equation has been calculated as y = mx + C. Whereas, y = Area under the peak or Response, m = Slope of the linear line (Constant), x = Concentration in µmL and C = intercept (Constant).

Fig. 3. Calibration curve of dl α-tocopherol acetate (96%) (Vitamin E).



HPLC is most widely used technique to analyze tocols, and both normal-phase (NP) and reversed-phase (RP) chromatography are applied (Kamal-Eldin *et al.,* 2000; Abidi, 2000; Ruperez *et al.,* 2001). Vitamin E functions as a chain-breaking antioxidant, neutralizing free radicals and preventing oxidation of lipids within membranes (McDowell, 2000). The lipophilic vitamin E has been detected in methanolic and acetone extracts of *H. longicorniculata.* Organic extracts of *H. longicorniculata* displayed significant antioxidant activity, proposed that the concentration of vitamin E might be higher along with the other natural antioxidants. The quantitative estimation of lipophillic vitamin E in tuber extracts of *H. longicorniculata* is depicted in Table 4. Methanolic extract showed highest concentration (2.7169 µg/mL) as compared to chloroform extract (1.9 µg/mL).

Fig. 4. Vitamin E standard peak at 10 µL.

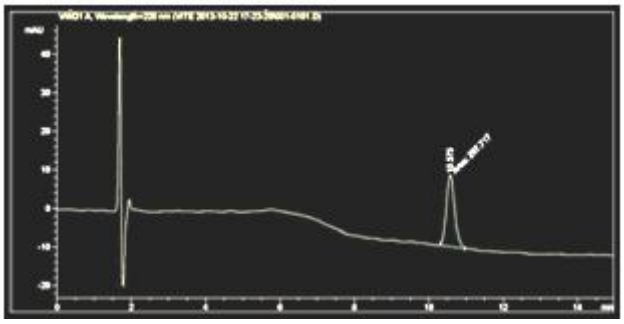


Fig. 5. Vitamin E standard peak at 25 µL.

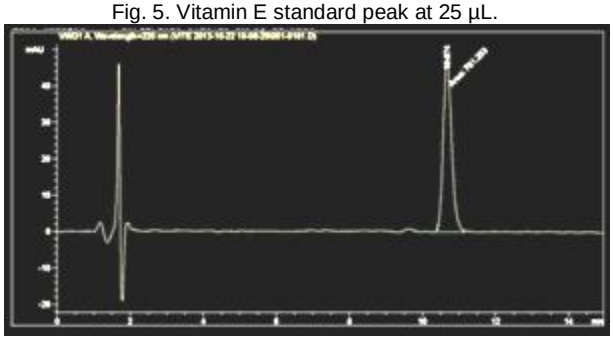


Fig. 6. Chloroform extract contains Vitamin E.

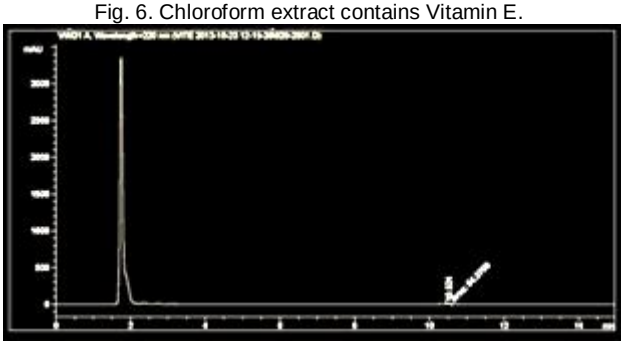
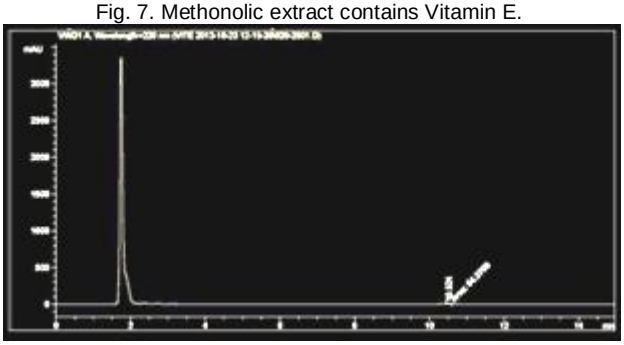


Fig. 7. Methonolic extract contains Vitamin E.

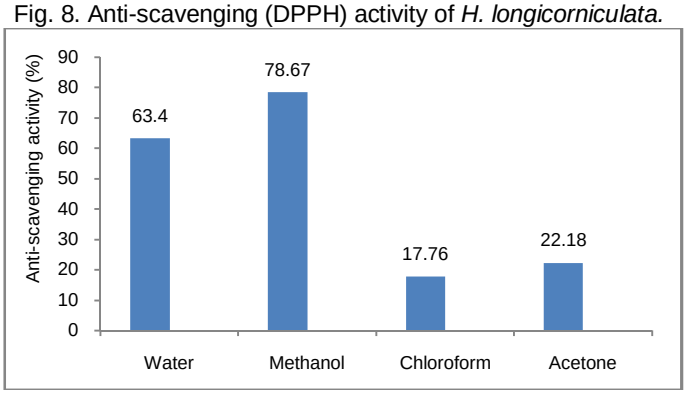


Vitamin C: The hydrophilic vitamin C (L-Ascorbic acid or L-ascorbate), an essential nutrient for humans and other animal species have been detected in aqueous tuber extract of *H. longicorniculata*. The vitamin C and β-carotene, which is the precursor for vitamin A, are important for absorption of some nutrients and eye vision. Therefore, the edible orchids can provide vitamin C for the people (elderly and refugees) who live in the areas where fresh fruits are limited (Kasulo *et al.,* 2009).

Table 4. Quantitative analysis of Vitamin E in all the plant extracts using y = mx + C.

Sr. No.	Sample name	Extract	Retention time (min)	Response	Concentration in µg/mL
STD	Standard sample dl a-tocopherol acetate	Methanolic solution	10.575	287.717	10
STD	Standard Sample dl a-tocopherol acetate	Methanolic solution	10.674	761.253	25
STD	Standard Sample dl a-tocopherol acetate	Methanolic solution	10.738	1594.99	50
STD	Standard Sample dl a-tocopherol acetate	Methanolic solution	10.719	3023.3	100
1	*Habenaria longicorniculata*	Chloroform	10.524	64.37	1.9
		Methanol	10.523	89.2445	2.7169

Fig. 8. Anti-scavenging (DPPH) activity of *H. longicorniculata.*



Anti-scavenging activity: Many reports are available on the protective effects of natural antioxidants against oxidative stress related disorders like ageing, degenerative diseases and cancer (Cozzi *et al.,* 1997). The phenolic compounds may have a direct contribution in antioxidant activity (Bidchol *et al*., 2011). The focal point of our present study was to explore the ethanopharmacological significance of genobiotic medicinal plants of Orchidaceae family possessing diversified chemical nature. Phytochemical screening of the crude plant extracts showed the positive reaction for alkaloids, flavonoids, phenolic compounds, saponins, glycosides, carbohydrates, vitamin C, vitamin E and minerals. The scavenging ability assayed is the ability of extracts to react rapidly with DPPH radicals and reduce most DPPH radical molecules. The antioxidant capacity of *H. longicorniculata* tuber extracts was measured by DPPH antiscavenging activity method and the results were expressed in Fig. 8. DPPH anti-scavenging activity of methanolic extract (78.67%) and water extract (63.4%) were comparable. Methanolic extract displayed significant antioxidant activity. These results might suggest higher medicinal suitability of alcoholic extracts in various antioxidant applications. The observations depicted in Fig. 8 suggested that the methanolic extract displayed highest antioxidant activity. The results showed a positive correlation of phenolic compounds, total flavanoids and vitamin E with antioxidant activity. On the other hand, a considerable DPPH radical scavenging activity was found in water extracts suggested the correlation of vitamin C and antioxidant activity.

Decline in activity in chloroform extracts was recorded, which conferred the presence of phenols and vitamin E at minimal concentration in the extract. However, acetone extract exhibited 22.18% antioxidant activity attributable to presence of phenols.

In vitro antimicrobial activity: All extracts were screened *in vitro* for their antimicrobial activities against clinically isolated bacterial and fungal strains such as *Staphylococcus aureus, Pseudomonas aeuroginosa, Salmonella typhi, Escherichia coli* and *Aspergillus niger.* It was found that the methanolic and aqueous extracts of the species displayed moderate activity against all the microbial strains. Whereas, the microbial strains were found to be highly resistant to acetone, chloroform and IPA extracts. The antimicrobial screening was carried out by disc diffusion method in respective solvents. The zone of inhibition was expressed in mm and compared with control solvents. The results are displayed in Table 5.

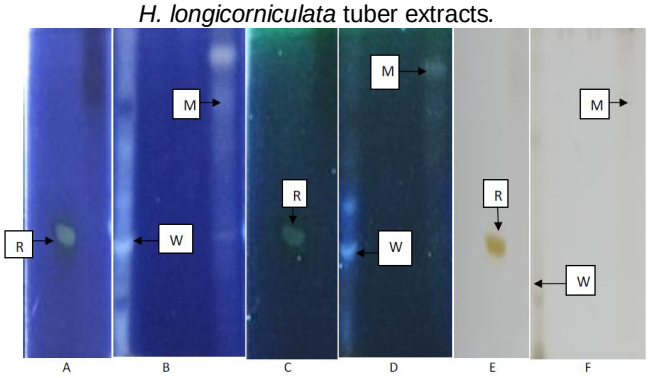
Flavonoids analysis by HPTLC: Flavonoids, the most important and most diverse natural phenolics (Agarwal, 1989) have diverse chemical and biological activities including radical scavenging properties. Figure 9 and 10 shows HPTLC profiles of aqueous, acetone, chloroform and methanolic tuber extracts of *H. longicorniculata.* In HPTLC techniques, the flavonoids from methanolic extracts were determined by using solvent system methyl acetate, acetic acid, formic acid and water (100:11:11:27) as a gradient mobile phase. In the chromatogram of *H. longicorniculata*, total 7 and 8 peaks were obtained at UV light 254 nm and 366 nm respectively as showed in Figs. 9 and 10. The Rf values were compared with standards and literature precedence. All values were good in conformity. The concentration of Luteolin (Rf = 0.33) was found abundantly, whereas, the flavonoids like Apiin (Rf = 0.40), Rutin (Rf = 0.47), Kaempferol (Rf = 0.89), Orientin (Rf = 0.71) were observed with moderate concentration in aqueous extract. The concentrations were confirmed by area under the peaks. The spots were analyzed under UV light 245 nm after derivatization. Some unidentified flavonoid-glycosides were present with significant concentration possessing low Rf values suggested that they have highest polarity.

Table 5. Antimicrobial activity of *H. longicorniculata* extracts.

Extract	*Salmonella typhi*	*Pseudomonas aeuroginosa*	*Staphylococcus aureus*	*Escherichia coli*	*Aspergillus niger*
Acetone	+	+	+	+	+
Chloroform	+	+	+	+	+
IPA	+	+	+	+	+
Methanol	++	++	++	++	++
Water	++	++	++	++	++

+ = 6-7 mm; ++ = 8-10 mm.

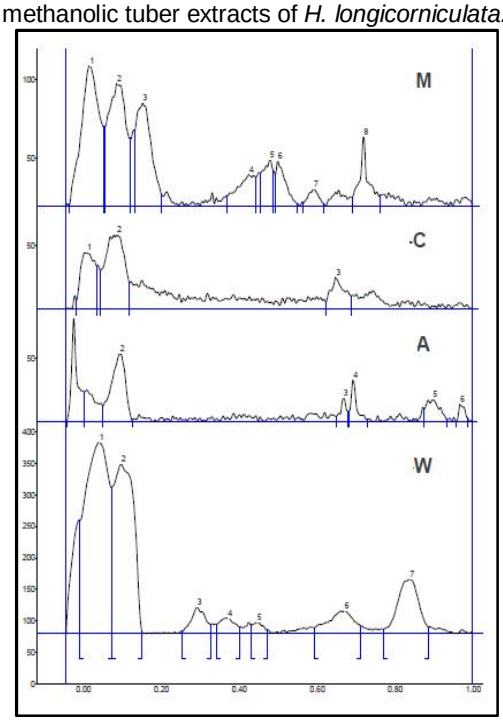
Fig. 9. HPTLC studies on flavonoids of *H. longicorniculata* tuber extracts.



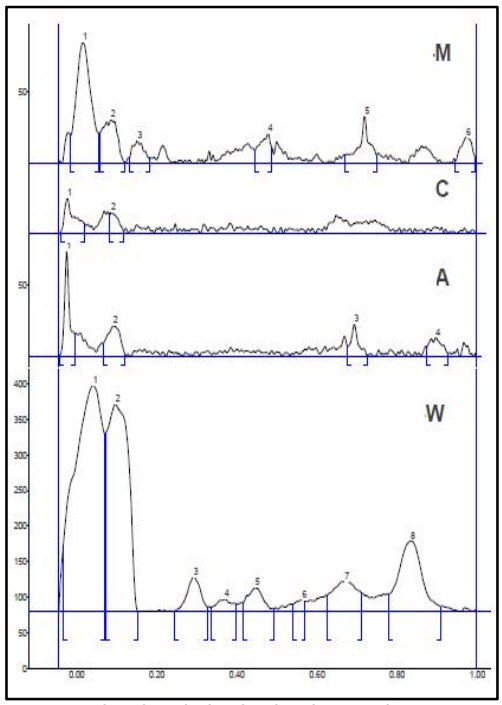
A. Standard Rutin under UV 254 AD
B. Water and Methanolic extracts under UV 254 AD
C. Standard Rutin under UV 366 AD
D. Water and Methanolic extracts under UV 366 AD
E. Standard Rutin under light
F. Water and Methanolic extracts under light

The TLC plate was screened under UV light 366 nm after derivatization. At 366 nm, additional spot of Coumaric acid (Rf = 0.91) was observed with moderate concentration (Fig. 9). The other spots of flavonoids identified at 254 nm were also located under UV 366 nm. The chromatogram of acetone extract was investigated at UV light. Totally 6 and 4 peaks were observed at 254 nm and 366 nm respectively. Two unknown flavonoids were observed whereas Astrangnlin (Rf = 0.68), Catechin (Rf = 0.96), Vanilic acid (Rf = 0.99), Isoquercetin (Rf = 0.72) were identified at UV light 254 nm and at 366 nm additional spot of Coumaric acid (Rf = 0.93) was observed. The two unknown peaks were located and peaks of Catechin, Astrangnlin and Vanilic acid disappeared in the chromatogram. Three peaks were observed in the chromatogram of chloroform extract at 254 nm. Among three peaks, two peaks were unidentified and peak of Astrangnlin (Rf = 0.69) was located in the chromatogram. Additional two peaks of unknown flavonoids were located at UV 366 nm. In methanolic extract, the chromatogram displayed 8 peaks, two remained unidentified while 6 were identified as flavonoids and phenolic acids. Saponanin (Rf = 0.22), Rutin (Rf = 0.45), Epigenin (Rf = 0.49), Chlorogenic acid, Cafeic acid (Rf = 0.76) were identified at UV 254 nm. At 366 nm, six peaks were obtained. Among 6 peaks, three unknown flavonoids have been located and a new peak of Vanilic acid (Rf = 1.00) was observed (Fig. 10). HPTLC peaks of standard and tuber extracts of *H. longicorniculata* is shown in Fig. 11.

Fig. 10. Peack diagrams of water, acetone, chloroform and methanolic tuber extracts of *H. longicorniculata*.



a. After derivatization (254 nm).

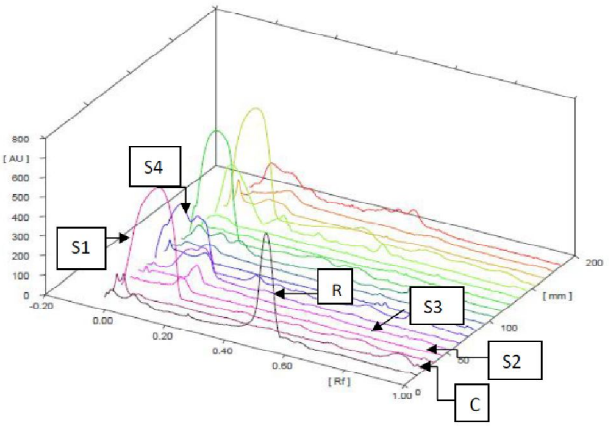


b. After derivatization (366 nm).

Table 6. HPTLC–Flavonoids profile of water, acetone, chloroform and methanolic tuber extracts of *H. longicorniculata* under UV 254 and 366 AD.

Sample extract	254 nm AD				366 nm AD			
	Rf	Height	Area	Assigned substances	Rf	Height	Area	Assigned substances
H. longicorniculata Water	0.07	231.9	14245.0	Unknown	0.07	250.0	17207.8	Unknown
	0.15	1.6	10879.4	Unknown	0.15	0.2	12067.9	Unknown
	0.33	14.4	1169.3	Luteolin	0.33	6.7	1280.5	Luteolin
	0.40	10.5	748.8	Apiin	0.40	11.1	530.3	Apiin
	0.47	5.6	375.2	Rutin	0.49	3.4	1012.7	Rutin
	0.71	12.0	1835.5	Orientin	0.57	14.4	310.4	Hespiridine
	0.89	10.1	3633.2	Kampeferol	0.71	25.6	2038.0	Orientin
					0.91	7.1	4466.2	Coumeric acid
H. longicorniculata Acetone	0.00	18.8	808.2	Unknown	-0.00	12.7	586.9	Unknown
	0.13	1.0	1228.5	Unknown	0.12	0.6	438.3	Unknown
	0.68	6.6	174.2	Apiin	0.73	0.3	258.2	Isoquercitin
	0.73	1.3	299.8	Isoquercitin	0.93	1.6	259.1	Catechin
	0.94	1.3	377.7	Catechin				
	0.99	2.5	133.3	Vanilic acid				
H. longicorniculata Chloroform	0.03	26.9	1012.5	Unknown	0.02	5.4	390.0	Unknown
	0.12	16.2	1835.6	Unknown	0.12	1.0	207.4	Unknown
	0.69	7.8	535.0	Apiin				
H. longicorniculata Methanol	0.05	50.2	3188.5	Unknown	0.05	17.4	1952.7	Unknown
	0.12	42.8	2775.8	Unknown	0.12	0.9	731.8	Unknown
	0.20	7.0	2015.6	Saponin	0.18	2.9	285.6	Unknown
	0.44	17.6	690.2	Rutin	0.49	9.1	357.2	Rutin
	0.49	22.4	585.9	Epigenin	0.75	4.8	532.4	Caffecic Acid
	0.55	1.4	550.7	Hespiridine	1.00	3.5	323.9	Vanilic acid
	0.62	1.6	217.2	Catechin				
	0.76	6.6	752.6	Caffecic acid				

Fig. 11. HPTLC peaks of standard and tuber extracts of *H. longicorniculata.*



All peaks at 254 AD (R-STD Rutin, C- Catechin, S1-Water, S2-Acetone, S3-Chloroform and S4-Methanol).

Conclusion

Habenaria species have an ancient history of the multiple indigenous uses and is one of the most highly commercialized indigenous traditional medicines from India. Investigations of phytochemicals and their biological activity have provided scientific support for many of its traditional uses.

An improved RP-HPLC-UV method has been successfully applied for determination of dl α-tocopherol acetate in organic extracts of *Habenaria longicorniculata*. Similarly the results obtained from phytochemical analysis illustrated the occurrences of various micronutrients i.e. carbohydrates, vitamin C, vitamin E, flavonoids, phenols, glycosides, saponins and minerals i.e. Zn, Cu, Mn, Fe. The present findings for microelements suggested that their contents are responsible for significant antioxidant activity in all extracts. The quantitative estimation of vitamin E also showed its role as a natural antioxidant. The antimicrobial activity of all the extracts did not show promising results against the clinically isolated microbial strains. The detection of flavonoids by HPTLC also revealed strong antioxidant activity in all the extracts. The structural characterizations (FTIR, NMR studies) of isolated flavonioids from various extracts of *H. longicorniculata* are in progress.

Acknowledgements

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