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Propagation and Phytochemical Analysis of *Crepidium acuminatum* (D.Don) Szlach.

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Abstract: *Orchidaceae is the second largest family of flowering plants with nearly 35,000 species in more than 850 genera. The whole family is endangered of survival due to habitat loss, fragmentation of populations, genetic drift and huge anthropogenic pressures. The Chinese were the first to cultivate and describe orchids for their medicinal uses. Theophrastus reported the medicinal properties of orchids. De Materia Medica of Dioscorides featured two terrestrial orchid species. Ashtavarga, the important ingredient of various classical Ayurvedic formulations, is a group of eight herbs, out of which four are orchids namely Malaxis muscifera, Malaxis acuminata syn. Crepidium acuminatum syn. Microstylis wallichii, Habenaria intermedia, Habenaria edgeworthii. In addition to these, Dactylorhiza hatagirea (Salam panja), Dendrobium macrei (Swarna Jivanti), Eulophia dabia (Salam misari), Eulophia nuda (Amarkanda), Vanda tassellata (Rasna), Saccolabium papillosum (Rasna substitute) are also acclaimed for their tremendous therapeutic potential. There have been a number of reports of their curative properties including diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, hypoglycemic, antimicrobial, anticonvulsive, neuro-protective and antiviral. These have been attributed to the presence of certain phytochemicals. Similarly, orchid seeds can be germinated in vitro without fungal association. Seed culture will help in the production of quality planting materials in larger scale. Orchids are mainly used as nutraceuticals due to the want of scientific studies on the chemical evaluation for drug and nutritional constituents and clinical safety and toxicity. Now when we are imparting the various herbal values of Himalayan flora, this is also the right time to know the medicinal value of the orchids. Initiative must be undertaken for Astavarga plants with germplasm collections and maintenance. Hence there is a dire need to study phytoconstituents, pharmacological evaluation and in vitro propagation of these orchids. Out of four orchids plants mentioned in 'Astavarga' we have concentrated on Crepidium acuminatum (D.Don) Szlach since a very less work is reported on this plant.*

I. Introduction

Nature is an agent for medicines. Uses of herbal remedies in healthcare preparations have been reported in Vedas and the Bible, highlighting the presence of natural products with medicinal properties in large number of wild plants. Plants produce a diverse group of bioactive molecules, making them a rich source of different types of medicines (Durga *et al.*, 2009). These days medicinal and neutraceutical herbs are receiving immense scientific attention for their holistic effects (Cousins *et al.*, 2009). Thus, natural products with pharmacological or biological activities are playing a very important role in medicine (Cragg *et al.*, 1997). World Health Organization (WHO) has confirmed that herbal medicines are serving the health needs of about 80 percent of the world's population especially in rural areas of developing countries. Attention has also been paid due to the side effects of most modern drugs. It has been estimated that in the mid-1990s over 200 companies and research organizations worldwide are screening plant and animal compounds for medicinal properties (Kong *et al.*, 2010; Manako *et al.*, 2001). Important drugs like vinblastine, vincristine, topotecan, taxol, teniposide, etopside, irinotecan etc. have come from plant sources. Curiosity is escalating in the fitness wellness profit of medicinal plants. Traditional plant medicine is becoming an area of ever-increasing importance in the health care systems. Since times immemorial, plants form the basis of various traditional therapeutic systems like, Ayurveda, Unanai, Sidha.

II. About Orchids

Orchids, belonging to the family Orchidaceae, have been known for their beauty and utility. In recent times, even though they are being exploited commercially more for their floricultural value, their therapeutic potential had been realised since ages.

There is need for propagation of the Orchids because orchids are out breeders, with habit and habitat specificity. They have complex life cycle with the presence of large number of seeds with reduced endosperm and development of a specific organ called the protocorm, during seed germination. They are dependent on symbiotic mycorrhizal association and intricate pollination mechanisms for continued reproduction and growth in nature. Natural populations of orchids are on a decline due to their inherent slow growing character and very low germination in nature. Additional commercial and anthropogenic pressures add to their duress and have thrown the whole family to the verge of extinction. Therefore, conservation strategies involving large scale multiplication of plants is required. Propagation *in vitro* can be judiciously exploited for restoration of these plants. Tissue culture techniques have opened newer possibilities for their commercialisation and conservation (Pathak *et al.,* 2011).

We find their reference as medicinal plants in old scriptures in Chinese and Indian systems. Indian Ayurveda is based on a group of 8 medicinal plants called the 'Ashtvarga'. Out of these, four plants are orchids namely, *Malaxis acuminata (Crepidium acuminatum), Malaxis muscifera, Habenaria intermedia* and *Habenariaedgeworthii*. They were known to impart Jeevaniya (Vitality), Vayashapan (revival of youthful conditions) body nourishment and antioxidant effects. The potential of orchids as therapeutic and neutraceutical agents in herbal formulations is being increasingly realised in the current times. Their potential is, however, not commensurate with the extent of research being done on them. Therefore there is need to explore these plants of 'Ashtvarga' for phytochemical constituents and *in vitro* propagation. For our research we have chosen *Crepidium acuminatum* (D.Don) Szlach.

Figure1. *Creipidium acuminatum* (D.Don) Szlach in natural habitat

Crepidium acuminatum* (D.Don) Szlach.** syn. ***Malaxis acuminata (Fragm, 1995) syn. ***Microstylis wallichii*** (Rastogi *et al.*1980-84) is distributed in Himalayas from Himachal Pradesh to Sikkim, Meghalaya, Peninsular India and Andaman islands, at altitude 450-3000m. (Rastogi and Mehrotra**,** 1980-84)

Syn. *Malaxis acuminata* D.Don(Fragm), *Microstylis wallichii* Lindl.(Rastogi and Mehrotra 1980-84).The accepted name is *Crepidium acuminatum* (Source: Kew Royal Botanic Garden http://apps.kew.org/wcsp/qsearch.do **)**

Lohani *et al.,* (2013) studied Chemical composition of *Microstylis wallichii* Lindl. from Western Himalaya They evaluated metal content and volatile constituents *in M. wallichii* collected from Uttarakhand by Atomic Absorption Spectrophotometer and GC and GC-MS respectively. Singh *et al.* (2009) reported Ayurvedic names for this plant as Jivaka, Chiranjivi, Dirghayu, Harsanga, Ksveda, Kurchasira, Pranda.

Sharma *et al.* (2009) studied the physicochemical variation of *Microstylis walichii.* They reported that standardization of the herbal drugs is essential for assuring the therapeutic efficacy of the herbal drugs. They evaluated the pharmacognostical as well as physico-chemical standards of *Microstylis wallichii* with emphasis on TLC fingerprinting of the drug for chemical identification.

Compounds reported from leaves of this plant are limonene,eugenol,citronellal,1,8-cineole,p-cymene,piperitone.β-Sitosterol,ceryl alcohol, glucose, rhamnose and choline have been reported from

pseudobulbs.(Rastogi & Mehrotra 1980-84) Sharma *et al. (*2009) studied the Physicochemical Variation of *Microstylis wallichii* .They evaluated the pharmacognostical as well as physico-chemical standards of *Microstylis wallichii* especially emphasis on TLC fingerprinting of the drug for chemical identification. Qualitative analysis was done for Tarikhet sample and Market sample. And reported most of plant metabolites wrer common except alkaloid and flavonoids which were absent in market sample. They discussed that chemical assay of *Microstylis wallichii* Lindl is however not established. They also reported chemical mapping of various extracts of this plant.

Chinmay *et al.* (2011) phyto pharmacognostical studies of *Malaxis acuminata and Malaxis mucifera.* They studied macro and microscopic characters of the two species.

Jeevak (*Malaxis muscifera* (Lindl.) Kuntze) and Rishibhak (*Malaxis acuminata* D.Don).They carried out pharmacognostical identity of wild sample and market samples used in the name of Jeevak and Rishibhak. They examined the macro and microscopic studies of these two species. They reported physic chemical parameters of these two plants and studied TLC fingerprint of 90% ethanol extract. They also reported distinguishing features of these two species.

Bhatnagar *et al.*(1984) chemical investigations on *Microstylis wallichi* and reported that pseudobulbs of *Microstylis wallichi* Lindl., an Indian drug used in 'Astavarga', were found to contain one sterol namely β–sitosterol, an alcohol identified as ceryl alcohol, two sugars namely glucose and rhamnose and five basic compounds one of them being choline. Jalal *et al.* (2008) discussed that a little attention has been paid on the plants of Astavarga.

Table 1. Phytochemicals Isolated from Orchids

Class	Phytochemical	Source	Reference
Alkaloids	Cephalandole	*Cephalanceropsis gracilis*	Wu *et al.*, 2006[a]
	Cremastrine	*Cremastra appendiculata*	Ikeda *et al.*, 2005
	Dendrobine	*Dendrobium nobile*	Kudo *et al.*, 1983
	Shihunidine	*Dendrobium lodigesii*	Li *et al.*, 1991
Bibenzyl derivatives	Alkyl ferulates	*Dendrobium moniliforme*	Lo *et al.*, 2004
	Gigantol	*Cymbidium goeringii* *Epidendrum rigidum* *Scaphyglottis livida*	Won *et al.*, 2006; Hernandez - Romero *et al.*, 2005; Deciga-Campos *et al.*, 2007[b]
	Cumulatin, Densiflorol A	*Bulbophyllum kwangtungense*	Wu *et al.*, 2006[b]
	Pholidotol A & B	*Pholidota chinensis*	Wang *et al.*, 2006[a]
	Aloifol	*Nidema boothii*	Hernandez-Romero *et al.*, 2004
Flavonoids	Derivative of Quercetin	*Anoectochilus roxburghii*	He *et al.*, 2006
	Quercetin	*Dendrobium tosaense*	Lo *et al.*, 2004
	Chrysin	*Cypripedium macranthos*	Shimura *et al.*, 2007
	Homoisoflavanone	*Cremastra appendiculata*	Fan *et al.*, 2001
Phenanthrenes	Coeloginanthridin	*Coelogyne cristata*	Majumder *et al.*, 2001
	Moscatin	*Dendrobium loddigesii*	Chen *et al.*, 1994
	Fimbriol A	*Maxillaria densa*	Deciga-Campos *et al.*, 2007[a]
Terpenoids	Dendroside A	*Dendrobium nobile*	Zhao *et al.*, 2001
	Dendromoniliside A & B	*Dendrobium moniliforme*	Zhao *et al.*, 2003

Sharma *et al.* (2007) examined the ethanolic (50% v/v) extracts of *Carissa carandas* (fruits) (Apocynaceae) and *Microstylis wallichii* (tubers) (Orchidaceae) for anti-inflammatory and analgesic activities in experimental animals. *Carissa carandas* and *Microstylis wallichii* (50 - 200 mg/kg) caused a dose dependent inhibition of swelling caused by carrageenin significantly in cotton pellet induced granuloma in rats ($P < 0.05$ to $P < 0.001$). There was a significant increase in the analgesy meter induced pain in rats. The extracts of *Carissa carandas* and *Microstylis wallichii* resulted in an inhibition of stretching episodes and percentage protection was 16.05 - 17.58% respectively in acetic acid induced writhing. There have been a number of reports on the biological activity of the phytochemicals. Lo *et al.* (2004) investigated free radical scavenging active components from *in vitro*propagated medicinal herbs of the genus Dendrobium. Fan *et al.* (2001) isolated benzoxides and phenanthrene from *Dendrobium densiflorum*. Five compounds were isolated on the basis of spectroscopic analysis, which are having anti-platelet activity. Gutierrez (2010) reviewed biological active compounds of medicinal orchids and their pharmacology. Chinsamy *et al*. (2011) reported biological activity and chemical composition of South African Medicinal orchids**.**

Table 2. Biological Activity of Bioactive Compounds in Orchids

Biological Activity	**Reference**
Antimicrobial	Shimura *et al.*, 2007
Anti inflammatory	Wang *et al.*, 2006[b]
Antioxidant	He *et al.*, 2006
Anticancer Activity	Wu *et al.*, 2006
Antipyretic	Chen and Chen,1935
Antimutagenic	Miyazawa *et al.1999*
Anticonvulsive	He *et al.*, 2006
Antihelmintic	*Rhee et al. 1982*
Antihepatotoxic	Wu *et al.*, 2007
Wound healing	Nayak *et al.*,2011
Anti platelet	Ding *et al.*, 2007
Antidiabetic	Wu *et al.*, 2004
Immunomodulatory activity	Ye *et al., 2002*
Pain relieving	Deciga-Campos *et al.*, 2007
Antivirus	Deciga Campos *et al.*, 2007
Herbicidal agent	Hermandez *et al.*, 2005

Aremongla *et al.,* (2012) studied germination of immature embryos and multiplication of *Malaxis acuminata* D. Don. They investigated immature seeds of 7-8 WAP were germinated on MS medium (3% sucrose) and used different combinations of NAA, BA and activated charcoal.75% plants survived. Dhayni *et al.* (2010) studied the Importance of Astavarga plants in traditional systems of medicine in Garhwal, Indian Himalaya whereas Cheruvathur *et al.,* (1971) studied adventitious shoot induction from cultured internodal explants of *Malaxis acuminata* D. Don

Abraham (2010) evaluated adventitious shoot induction from cultured internodal explants of *Malaxis acuminata* D. Don .Plants were grown on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (Kn), and thidiazuron (TDZ). Of the three cytokinins used, TDZ at 3 mg/L induced the highest frequency (82%) of organogenic explants. However, all responding explants produced only a single adventitious shoot irrespective of the type and concentration of the cytokinin. Adding 0.5 mg/L naphthaleneacetic acid (NAA) to the medium enhanced adventitious shoot formation. In the presence of 3 mg/L TDZ and 0.5 mg NAA, frequency of organogenesis was 96% with a mean number of 6.1 shoots per explants

Kaur *et al.,* (2009)[a] micro propagated *Malaxis acuminata* and studied its regeneration potential of pseudo bulb, Plants were procured from *in vitro* grown cultures, was analysed in Mitra medium and its combinations with growth adjuncts.. The explants from relatively older pseudo bulbs (>0.5cm in length) remained recalcitrant to regeneration whereas those representing younger ones (<0.5cm in length) responded positively. Shoot buds were induced with growth regulators (BAP/NAA; 1mg/L each) whereas their combination [BAP (1.0 mg/L) + NAA (1.0 mg/L)] promoted protocorm-like body formation in the explants. Additional activated charcoal proved beneficial in accelerating the morphogenetic processes leading to plantlet development. NAA (1 mg/l) impaired the response frequency and delayed subsequent morphogenetic processes leading to plantlet development. BAP+NAA (1.0 mg/l) +AC (2 g/l) was used best for early initiation, highest regeneration frequency, proliferation of protocorm-like bodies (PLB), and plantlet development. Plantlets were transferred to clay pots containing potting mixture (sand, soil, leaf compost) in the ratio of 1:1:1. Nearly 70-80% of plantlets survival was recorded. Histological investigation confirmed that neo formations were dermal and multi-cellular in origin

Da Silva (2013) reviewed orchids in terms of tissue culture, genetics, phytochemistry and transgenic biotechnology till 2005.He mentioned regeneration, development, cell, tissue, organ culture, micro propagation, seed germination and conventional breeding in a number of orchid plants.

Kaur *et al.* (2009)[b] studied *in vitro* propagation of *Vanda testacea* (Lindl.) Reichb.f. foliar explants cultured on Mitra (M) medium with 1.0 mg/l BAP, Kn each and 1.0 mg/l NAA individually and in combination for initiation of regeneration response, proliferation of regenerants and subsequent development of plantlets. Juvenility of the tissues and chemical stimulus were important factors in initiating the regeneration response in the explants. The relatively older leaf explants (>1cm in length) remained recalcitrant to regeneration \whereas when used withBA and NAA, the explants showed callus proliferation and further differentiated into PLBs. An individual treatment with NAA (1.0 mg/l) impaired the response frequency and delayed further morphogenetic processes leading to plantlet development. The best response in the explants (in terms of high regeneration frequency, early initiation, PLB proliferations, and plantlet development) was observed in 1.0 mg/l BAP alone/with 1.0 mg/l NAA + activated charcoal. Plantlets were transferred to pots containing epiphytic compost .Nearly 75% of plantlets survival was recorded.

Giri *et al.* (2012) harnessed the total phenol and phenolic compounds of *Habenaria edgeworthii* by using seed derived callus. They also formulated an efficient *in vitro* propagation protocol for the species where bioactive compounds were enhanced by different tissue culture techniques. The effect of 6-benzyladenine (BA) and methyl jasmonate (MeJA) on growth of callus suspension cultures was studied. Phenol content was determined by Folin-Ciocalteu's calorimetric method. They observed that the total phenol content increased with increasing BA concentration. Hossain *et al.* (2013) designed protocol for *in vitro* mass propagation of *Cymbidium aloifolium* by using different types of media like Murashige and Skoog (MS), Phytamax (PM), Mitra *et al.* (M) and Knudson C (KC), were evaluated for seed germination and early protocorm development in *Cymbidium aloifolium*, a medicinally important plant having bibenzyl dihydrophenenthrene which are biologically active phytochemicals. Effect of peptone, activated charcoal, plant growth regulators, 6-benzyladenine and 2,4-dichlorophenoxyacetic acid (1.0-2.0 mg/L), and light and dark conditions were studied. Mitra medium supplemented with 2.0 g/L activated charcoal (AC) showed 100% seed germination and effective for induction of significantly large size protocorms. Pathak *et al.* (2011) worked on *Gastrochilus calceolaris* where *in vitro*asymbiotic seed germination potential of its immature seeds was tested on different media. They developed a propagate system for mass multiplication of this species. Mahendran *et al.* (2009) established a method for mass multiplication via seed culture in *Satyrium nepalense*, a medicinal orchid used as an energizing tonic, and treatment for malaria and dysentery. Cousins and Adelberg (2009) discussed various *in vitro* plant tissue culture techniques of medicinal plants and its industrial scale up for great economy and high plant quality. Panwar *et al.* (2012) propagated *Eulophia nuda* by *in vitro* techniques of plant tissue culture and developed a protocol for mass production and germplasm conservation. Vendrame *et al.* (2011) evaluated the effect of phloroglucinol in Green House for regeneration and survival of cryopreserved *Dendrobium nobile* protocorms. Nge and co-workers (2006) used Chitosan as a growth stimulator in Orchid tissue culture. They showed that a minor amount of chitosan has an insightful effect on the growth and development of orchid plant tissue. Rao and Ravishankar (2002) published a review on production of high-value secondary metabolites through biotechnological means which have value in pharmaceuticals and food additives by plant cell cultures, shoot cultures, root cultures and transgenic roots. Hossain *et al.* (2013) reviewed that orchid improvement through biotechnology for commercial purposes is well within reach and is being exploited for medicinal, nutraceutical and culinary uses. T. Arenmongla & Deb C.R. (2012). studied *in vitro* propagation of *Malaxis khasiana* through immature seed culture They *r*apidly propagated this plant by taking seeds of 8-9 weeks after pollination9WAP) cultured on MS medium by using 2% sucrose supplemented with 500 mg/l casein hydrolysate 1µM BA exhibited germination of 75% seeds after 107 days.

III. Conclusion

This plant belonging to the family Orchidaceae is one of the important sources of new bioactive compounds. From traditional ethnobotany to the highly sophisticated drug discovery approaches may be useful in this regard. Here we compiled the phytochemical and in vitro potential of *Crepidium acuminatum* (D.Don) Szlach an important medicinal plants of family Orchidaceae .A little work has been done on this plant. Hence future research should be focused on the isolation and identification of active compounds with pharmacological activity. In addition research should take in depth studies to know in vitro propagation techniques employed in this plant.

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