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Comparative Study of the Chemical Components of *Anoectochilus Roxburghii* and *Anoectochilus Formosanus* Tissue Culture

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Abstract**: Anoectochilus roxburghii* **(Wall.) Lindl. is a herbaceous epiphytic orchid that is found in the following locations: Yunnan, China; Assam, India; Bangladesh; Eastern Himalayas; Nepal;Western Himalayas; Sri Lanka; Myanmar; Thailand; and Vietnam.A.roxburghii plant provides valuable medicinal material.** ***A. formosanus **is a terrestrial orchid that is widely distributed in Taiwan and Fujian Province of China and in Japan.** ***A.roxburghii*** **has been used as a Chinese folk medicine for many years. However, few reports on its in vitro regeneration are available. Thus, expanding** ***A. roxburghii*(Wall.) Lindl. sources is important. In this paper, a rapid micropropagation and cell culture system was established for wild** ***A.roxburghii*** **harvested from Yunnan. We designed an optimized induction medium for protocorm-like bodies, and the medium comprised the following: Murashige and Skoog(MS) medium +2,4-dichlorophenoxyacetic acidat 1 mg/L + kinetinat 0.5 mg/L+1-naphthalene acetic acidat 1 mg/L+6-benzyladenineat 0.5 mg/L+1% agar+3% sucrose. This medium was tested and was shown to be equally suitable for shoot culture after 8 weeks. The culture was maintained up to 12 weeks. Other cultures were initiated from shoots inoculated on MS medium supplemented individually with different concentrations of hormones. Medium supplemented with 3% (w/v)sucrose was the best among all treatments.The major bioactive compounds of** ***A.roxburghii*** **and** ***A. formosanus*** **were polysaccharides, amino acids,and alkaloids, and these compounds were obtained from whole plants of both species. The dried and powdered extracts fromwhole plantswere obtained by ethanol extraction using Soxhlet method. When whole plants were subjected to ethanol extraction,the polysaccharide, amino acid, and alkaloid levelsin** ***A.roxburghii*** **were 5.2%,8.2%, and 3.8% and those in** ***A.formosanus*** **were 6%–8%,4.7%, and 7.6%, respectively.**

Keywords: **In vitro culture, Orchids,** ***Anoectochilus roxburghii*****, Orchidaceae, Natural products,*****Anoectochilus formosanus.***

I. INTRODUCTION

Anoectochilus is a genus that comprisesabout 50 orchids (family Orchidaceae) and belongs to the subfamily Orchidoideae(Zhang, 2007).A.roxburghii is distributed throughout southern China, Japan, Sri Lanka,India, and Nepal. A.roxburghii (Wall.) Lindl. is a rare and valuable perennial herb. Southern China's Guangxi, Guangdong, Guizhou, Sichuan, and Yunnan provinces have rich resources of medicinal herbs (Delectis Florae Reipublicae, 2000), and A. formosanus is one of four species of this genus found in Taiwan (Liu and Su, 1978). A. formosanus is distributed at elevations between 800 and 1,500 m in the central mountain range and Lanyu Islandin Taiwan, on Ryukyu Island in Japan, and in China's Fujian Province (Anon, 1999).

A. roxburghii (Wall.) Lindl., an Orchidaceae phanerophyte,is a well-known traditional Chinese herb(Shih, 2002). This genus is distributed at elevations between800 and 1800 meters as a creeping, ascending, terrestrial orchid that grows in warm to cool temperatures on rich humus in damp crevasses;the plant has gold leaves that are purple black on the underside with subcordate to ovate-acute shape, velvety,dark, lime-green,and reticulated (Ensminger, 1983). A. roxburghii is distributed throughout Taiwan, China, and Japan and is usedas awhole plant for treating fever, pleurodynia,

snake bite, lung and liver disease, hypertension, and malnourishment among children (Zhang, 1999). The habitat of this species is terrestrial, and the whole plant is used to treat tuberculosis (Agricultural Science Paper, 2013). This species has been studied for its medical uses, such as treatment of many diseases (e.g.,hypertension)(Chen, 2000), cancer (Lin, 1993) and as an antiviral drug (Shih, 2002), as an antidiabetic drug(He, 2004), as a treatment for liver disease (Huang, 2007), as a treatment for cardiovascular disease (Huang, 2008), and as a treatment for nephritis (Du, 2000). Studies have been conducted to investigate the propagation of this species by tissue culture techniques and cultivation.A. formosanus Hayata, a Chinese herb, is a valued folk medicine for fever, pain, and diseases of the lung and liver. Allergic asthma is characterized by increased serum IgE level and inflammation of the airways with high levels of interleukin (IL)-4 and IL-5 in bronchoalveolar lavage fluids. Constriction of airway smooth muscle and development of airway hyperresponsiveness are the most important symptoms of allergic asthma. In Taiwanese folk remedies, the whole plant of A.formosanus(fresh or dried) is boiled in water and consumedasa treatment for chest and abdominal pains (Hu, 1971), diabetes, nephritis (Chiu and Chang, 1995),fever, hypertension, impotence, liver and spleen disorders, and pleurodynia. The fresh herb is applied externally for snakebites (Kan, 1986). A. formosanus contains substances that affect arachidonic acid metabolism, which is involved in the function of the cardiovascular system (Mak et al., 1990; Huang et al., 1991). The aqueous extract of A. formosanushasanti-viral (Chan et al., 1994), anti-inflammatory, and liver-protective properties (Lin et al., 1993). In our previous study, a standardized aqueous extract ofA. formosanus was used to modulate innate immunity of normal mice. In this study, the effects of the extract on airway inflammatory infiltrations, including T cell differentiation, cytokine modulation, allergic antibodies estimation, pulmonary pathology were determined (C-C.Hsieh,H.B.Hsiao,W.B,Li, 2010). However, chemical components of A. roxburghiihave not been reported yet(Li.1995, Journal of Anhui Agricultural Sciences, 2009). A study was conducted to determine the optimum medium for adventitious buds; the optimum medium was found to be MS(Mushirag and Skooge)+ 6-benzyladenine (6-BA)at 1.0 mg/L+2,4-dichlorophenoxy acetic acid (2,4-D)at 0.1mg/L+3% saccharose+7 g/L agar(Fan, 1997). Many studies have focused on the *tissue culture conditions for this species because natural sources of A. roxburghiiare depleted (Guo, 2000;Yu, 2000;Pant,* 2013).A. formosanus Hayata belongs to the genus of Anoectochilus (family Orchidaceae). After surface sterilization, aboveground parts of A. formosanus werecollected from Fujian Province, China, and stems with axillary budsmeasuring about 1 cm were cut into small pieces and cultured in sterile micropropagation medium (MM) containing Murashige and Skoog (MS; Murashige and Skoog,1962) basic medium and supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA), 1 mg/L 6-benzyl adenine (6-BA), 1 g/L activated charcoal, and 30 g/L sucrose. The medium was solidified with 10 g/L agar and then incubated at 25±2 °C under a 12h photoperiod and a photosynthetic photon flux density of 50 μmol/(m2·s) (Zhang et al., 2010).The aim of the present study was to establish an effective method for A. roxburghii tissue culture and to compare thepolysaccharide, amino acid, and alkaloid levelsinwhole plantsofA.roxburghii and A. formosanus Hayata subjected to ethanol extraction.

II. MATERIALS AND METHODS

A. Tissue culture forA. Roxburghii:

In this study, we used MS medium as basic culture medium. The effects of 2,4-D, kinetin (KT),NAA,6-BA, and zeatin (ZT) on callus induction were investigated, and the induction process was conducted in the dark at 25 °C. Twenty segments were inoculated for each experiment. The experiment was repeated thrice using different hormone concentrations. To adapt to the final consumption demand of *A. roxburghii* cell culture, the subculture medium was adjusted to produce a subculture medium 2(SCM2), and the multiplication cycle was extended to 12 weeks.

Subculture medium 1 (SCM1) For experiment 1:MS +2,4-D at 0.5 mg/L+KT at 0.25 mg/L+NAA at 0.5 mg/L+6-BA at 1 mg/L+0.6% agar+9% sucrose.

Subculture medium 2 (SCM2) For experiment 2:MS +2,4-D at 1 mg/L+KT at 0.5 mg/L+NAA at 1 mg/L+6-BA at 0.5 mg/L+1% agar+3% sucrose.

Subculture medium3(SCM3) for experiment 3:MS+2,4-D at 0.2 mg/L+NAA at 0.9 mg/L+6-BA at 1mg/L+ZT at 0.25 mg/L+0.6%agar+4.5% sucrose.

B. Polysaccharides extraction and analysis:

About 1g of sample was collected and dried in a baking oven at55 °C for 20 h and then powdered using amortar. The powdered samples were treated with 10 mL petroleum ether and ultrasonicated for 10 min. The supernatant was removed,

and 20 mL ethanol solution was added to re suspend the samples. The samples were ultrasonicated for 10 min. The solvent was dried by rotary evaporator (57 °C), and 100 mL distilled water was added to the dried solvent. The solutions were kept in a water bath for 3 h at 90°C. Solutions were then filtered, and water was added to reach 100 mL total capacity. Filtrate (1 mL) was transferred to a 10 mL centrifuge tube, 5mL anhydrous ethanol was added, and the solution was mixed by shaking. The solution was placed at4 °Cin the refrigerator for 1 h and then centrifuged (6000 r/min) for 20 min. We added 5 mL of 80% ethanol and then kept the solution in the refrigerator at 4 °C for 20 min. The solvent was dried with N2 gas for 30–50 min, and drying was repeated once. Finally, 10 mL hot water (100 °C) was added to the solvent to dissolve the material, and the solutions were transferred to 25 mL volumetric flasks. Water was added to achieve the final volume. Each solution at 1 mL was mixed with 1 mL of 5%phenol.Sulfuric acid at 5 mLwas added, and the absorbance was measured at 490 nm wavelength with a UV spectrophotometer.

C. Amino acid extraction and analysis:

Samples at approximately 0.25 g were dried in an oven at 55 °C and powered. The powdered samples were transferred into plugged tapered bottles. Added 20 mL of 0.1mol/L HCl solution, and then ultrasonicated the sample at 30 °C for 30 min. We suction-filtered the mixture, and pH was then adjusted to neutral by using a15% sodium solution. Samples were transferred into 100 mL volumetric flasks, and a constant volume of 100 mLwas maintained using distilled water. The extract (2.0 mL) was placed into a 10 mL tube, and CH3COONa buffer salt solution (pH 6.5) at 1.0 mLwas added. Afterward, 1.0 mL of 2% ninhydrin solution was added. The mixture was placed in a boiling water bath for 40 min and then cooled for 15 min.The contents were measured under 568 nm with corresponding reagent blank. Aspartic acid (HPLC≥98%, CAS: 5794-13-8) was used as standard.

D. Extraction of the alkaloids from A. Roxburghii and A.formosanus:

We accurately weighed 1.0g of *A. roxburghii* and *A. formosanus* dry powders. Placed the powders from each species in a 100mLground flask. We added 50mLof extraction solvent(chloroform :methanol: ammonium = 15:5:1).We cold-soakedthe solutions overnight for 12h and treated them with ultrasound for 40min. Subsequently, we cooled the solutions to room temperature ,centrifuged them for 20min at a speed of 5000rpm. The supernatant fluids arerotary evaporated to dryness. The residues were dissolved with 10.0 mLof 2% H2SO4 solution and filtered using quantitative filter paper. Washed the filters and residue with 2.0 mL of 2% H2SO4solution and then with more than 10 mL of citric acid-sodium citrate buffer solution(0.1 mol·L-1). We adjusted the pH level of the filtrates to pH 5.4 and diluted each filtrate with buffer solution of pH5.4 up to a volume of 50mLfor later use. Accurately weighed 0.012g of Aconitum alkaloid, which was dissolved in10 mLof ethanol, and then transferred the mixture into 100mLvolumetric flasks. Water was added tovolume until a concentration of120 mg·L-1 was obtained .Precisely 1, 2, 3, 4, and 5 mLof Aconitum standard solution was measured, and water (5mL) was added. Citric acid-sodium citrate buffer solution (pH5.4; 1.0 mol·L-1) at 5 mL and 0.1% bromothymol blue solution at 1mL were added. Chloroform (10 mL) was added. After shaking for 2 min ,the solution was left to stand for 30min, and the chloroform layer was separated. Then, 0.5 g of anhydrous sodium sulfate was added to the chloroform layer. The mixture was shaken and left to stand for 30 min for later use. A1mLsolution of chloroform layerwas accurately measured in a 10ml volumetric flask, diluted with chloroform to volume, and mixed. A 5 mL buffer solution (normal control) was measured and subjected to the same procedure. Absorbance was measured at 412 nm.

TABLE I: rates of contents in *A. roxburghii* and *A. formosanus* extractions

Contents for *A.formosanus*	Contentsfor *A.roxburghii*	Materials
6%–8%	5.2%	polysaccharides
4.7%	8.2%	amino acids
7.6%	3.8%	alkaloids

III. RESULTS

A. The effects of different concentrations of hormones on growth of A. roxburghii Wall. Lindl:

The effects of different concentrations of hormones on the growth of (*A. roxburghiiWall* Lindl.)were investigated. Molecular approaches can positively contribute to the development of cell, tissue, and organ culture systems during in

vitro growth and regeneration of medicinal plants. In addition, tissue culture systems could prove useful for large-scale biotechnological production of medicinal plant phytochemicals (Pretto, 2000). Furthermore, uniform plant growth with consistent plant material can be achieved. Plants can be grown under sterile and standardized conditions and are free from biotic and bioticcontamination. This study describes the basic procedures to induce multiple shoots or explants of *A. roxburghii.* Formation of new shoots was observed in all media studied except in the control group (in hormone-free medium), thereby indicating that *A. roxburghii* is highly responsive to plant growth regulators. Regeneration frequency and mean number and length of shoots per explants were recorded after all hormone experiments. In the first stage of our experiment, the number of shoots changed depending on the different concentrations of hormones. In MS1, shoots were used, and the growth was poor and very slowly (Fig. 1). All the investigated concentrations of KT promoted shoot production. However, the best result was obtained with KT at a concentration of 0.5mg/L and when explants were used in MS2 medium. Based on the results, the number of shoots increased with increasing KT concentration. However, smaller and shorter shoots were formed with decreasing KT concentration in the culture medium. Excessive shoot length and root formation were observed (Fig.2). Effective extraction was observed in the presence of 2,4-D,NAA, and 6-BA in the medium, which also increased shoot length. By contrast, in MS3medium, ZT increased the number of shoots (Fig.3).

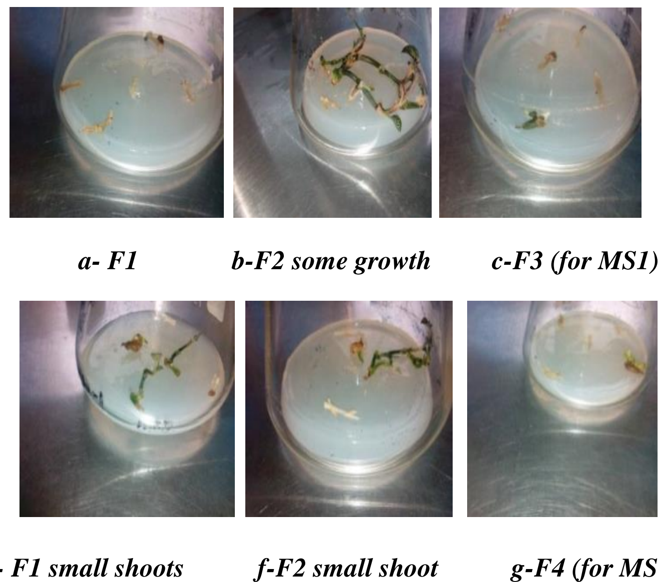

Figure: 1. Tissue culture for *Anoectochilus* shoots in MS1 medium

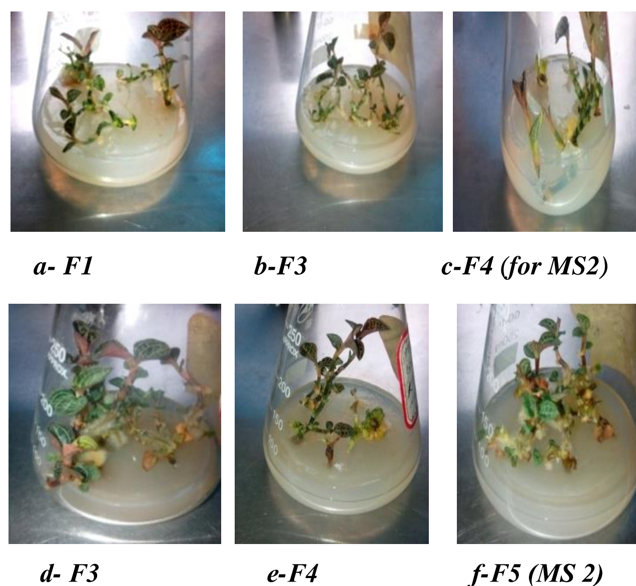

Figure 2.Effect of KT hormone on tissue culture of *Anoectochilus* explants in MS2 medium.

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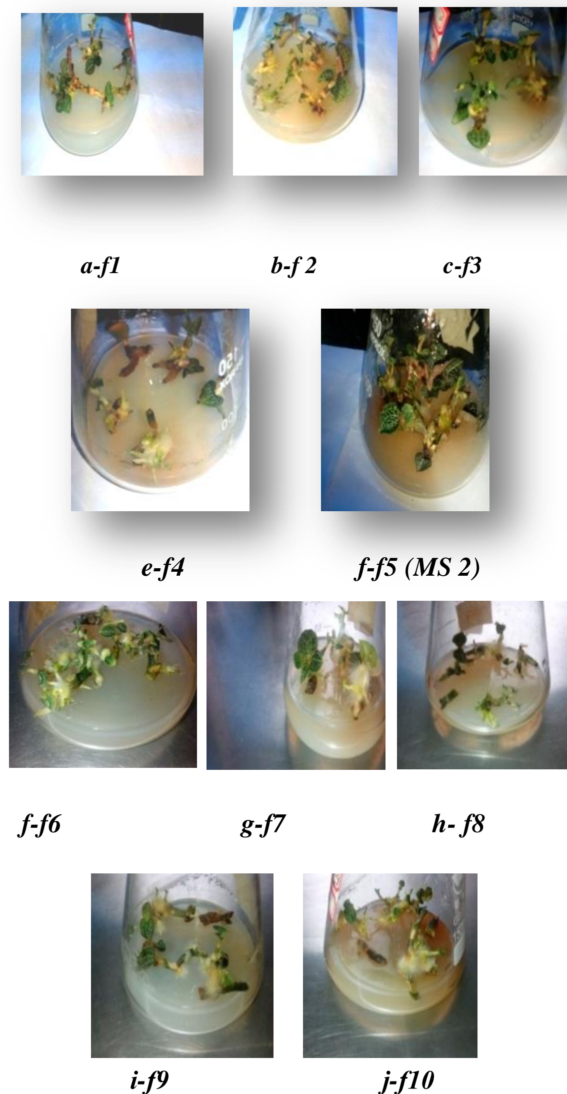

Figure.3. Effect of ZT hormone on *A. roxburghii* culture in MS3 medium Note :(a–e) for explants; (f–j) for stems.

Table II. Effect of sucrose concentration on the growth of orchids

Sucrose (%)	Average number of shoots/explants (Mean ± SE)	Average length of shoots (cm) (Mean ± SE)
9%	MS1(0–3)	1–5cm
3%	MS2(1–4)	0.3–6cm
4.5%	MS3 (1–10)	0.5–4cm

B. Effect of different sugars on the growth of orchids (A. roxburghii):

Protocorm growth and development were greatly affected by the source and concentration of sugars added to the culture media (Fig. 2). Shoot, root, and leaf formations were significantly enhanced with decreasing sugar concentration. Medium supplemented with 3%(w/v) sucrose was the best among the media and yielded 1±4 shoots/explants with a shoot length of0.3 ± 6cm. Additionally, sugar at a lower concentration had a good effect on the physiology, growth, and differentiation of cultured cells and tissues.

C. *The effects of different concentrations of KT on the growth of orchids (A. roxburghii):*

All the investigated concentrations of KT promoted shoot production. However, the best result was obtained when the medium was supplemented with KT at a concentration of 0.5 m/L in MS2. The number of shoots increased with

increasing KT concentration. However, smaller and shorter shoots were formed as the concentration of KT decreased in the culture medium. Excessive shoot length and root formation were observed.

IV. CONCLUSION

In conclusion, MS2 containing3%w/v sucrose was the best medium for the growth and multiple shoot induction of orchids (*A. roxburghii*) cultured in vitro. The best result was obtained with KT at a concentration of 0.5mg/L and when explants were used in MS2 medium. Based on the results, the number of shoots increased with increasing KT concentration. However, smaller and shorter shoots were formed with decreasing KT concentration in the culture medium. Excessive shoot length and root formation were observed (Fig.2). Medium supplemented with 3%(w/v) sucrose was the best among the media and yielded 1±4 shoots/explants with a shoot length of0.3 ± 6cm. Additionally, sugar at a lower concentration had a good effect on the physiology, growth, and differentiation of cultured cells and tissues.

When whole plants were subjected to ethanol extraction, the polysaccharide, amino acid, and alkaloid levels in *A. roxburghii* were 5.2%, 8.2%, and 3.8% and those in *A. formosanus* were 6%–8%, 4.7%, and 7.6%, respectively, as shown in Table(1).

REFERENCES

[1] Zhang Zhong, Deng Lin Qiong, Li-Hua Huang, et al. A. roxburghii Research Status and Prospects [J]. Guizhou Science, 2007,25 (2): 81.

[2] Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita(2000). Flora Reipublicae Popularis Sinicae, Tomus 27. Science Press, Beijing (in Chinese with an English abstract).

[3] Shih, C. C., Wu, Y. W., Lin, W. C., Clin. Exp. Pharmacol.Physiol. 2002, 9, 684–688.

[4] Ensminger A.H., Ensminger, M. E., Konlande, J.E., and J.R.K. Robson, J.R.K.(1983). Food & Nutrition Encyclopedia. Vol. 2, pp. 1427-1441, Pegus Press,Clovis, California, U.S.A.

[5] Zhang JH, Guo SX, Yang JS (1999). Studies on chemical constituents of the fungus accelerating the growth of Anoectochilus roxburghii. Chin Pharm J 34, 800–802 (in Chinese with an English abstract).

[6] Agricultural Science Paper, 2013.Dynamic Change of Main Active Ingredients in A. roxburghii (Wall) Lindl and A.formosanus Hayata in Different Growth Stages.

[7] Chen, Z., Huang, Z. Q., J. Fujian Med. Univ. 2000, 34,350–354.

[8] Lin, J. M., Lin, C. C., Chiu, H. F., Am. J. Chin. Med. 1993,21, 59–69.

[9] Shih, C. C., Wu, Y. W., Lin, W. C., Am. J. Chin. Med 2002, 31, 25–31.

[10] He, C. N., Wang, C. L., Ge, S. X., Chin. Phom. J. 2004, 39,81–85.

[11] Huang, L. Y., Chen, T. W., Ye, Z., Chen, G. N., J. Mass Spectrom. 2007, 42, 910–917.

[12] Huang, L. Y., Cao, Y. Y., Chen, G. N., Sep. Purif. Technol.2008, 64, 101–107.

[13] Du, X. M., Sun, N. Y., Irino, N., Shoyama, Y., Chem.Pharmaceut. Bull. 2000, 48, 1803–1809.

[14] Li M, Zou D (1995). Study on pharmacology activity of Anoectochilus roxburghii from three sources. Information Chin. Pharmacol. Soc. 3:26-28.

[15] Journal of Anhui Agricultural Sciences,2009-03.Induction of Adventitious Buds of Anoectochilus roxburghii KAN Shi-chao et al. (Collage of Life Science, Guizhou University Guiyang. Guizhou.

[16] Fan ZN, Xiao HS, Fan XH, Wu WS (1997). Study on tissue culture of Anoectochilus roxburghii. Journal Fujian Normal University (Nat Sci Ed) 13: 82-87.

[17] Guo SX, Chen XM, Yu XM, Fan L (2000). Investigation on the isolation of myconhizal fungi from Anoectochilus roxburghii and its biological activity. Chin Pharm J 35, 443–445 (in Chinese).

[18] Yu XM, Guo SX(2000). Establishment of symbiotic system for Anoectochilus roxburghii (Wall.) Lindl. and

endophytic fungi. J Chin Materia Med 25, 81–82 (in Chinese with an English abstract).

[19] Pant B., 2013.Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. Plant Biotechnology and Biochemistry Laboratory, Central Department of Botany, Tribhuvan University, Kathmandu, Nepal.

[20] Pretto F.R. and Santarem, E.R. (2000). Callus formation and plant regeneration from Hypericum perforatum L. leaves. Plant Cell Tissue and Organ Culture 67:107-113.

[21] Anon. 1999. Anoectochilus formosanus Hayata. In “Chung-HuaBen-Tsao”,Vol. 8, Shanghai Science Technology Press,Shanghai, pp. 672-673. (in Chinese)

[22] Liu, T.S. and H.J. Su. 1978. Anoetochilus formosanus Hayata,In H.L. Li, T.S. Liu, T.C. Huang, T. Koyama, and C.E.Devol (eds.), Flora of Taiwan, vol. 5. Epoch Publishing Co.,Ltd., Taipei, Taiwan, pp. 874-876.

[23] C-C.Hsieh,H.B.Hsiao,W.B,Lin(July 2010). A standardized aqueous extract of Anoectochilus formosanusmodulated airway hyperresponsiveness in an OVA-inhaled murine modelVolume 17, Issues 8–9, Pages 557–562

[24] C-C.Hsieh,H.B.Hsiao,W.B,Lin(July 2010). A standardized aqueous extract of Anoectochilus formosanusmodulated airway hyperresponsiveness in an OVA-inhaled murine modelVolume 17, Issues 8–9, Pages 557–562

[25] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures.Physiol Plantarum. 1962;15(3):473–497.doi:0.1111/j.1399-3054.1962.tb08052.x. [Cross Ref]

[26] Zhang FS, Lv YL, Dong HL, Guo SX. Analysis of genetic stability through intersimple sequence repeats molecular markers in micropropagated plantlets of Anoectochilus formosanus HAYATA, a medicinal plant.Biol Pharm Bull. 2010;33(3):384–388.doi:10.1248/bpb.33.384. [PubMed] [Cross Ref]

[27] Fujian Institute of Traditional Chinese Medicine. Record of Fujian Materia Medica. Fuzhou, China: Fujian Science and Technology Press; 1982. (in Chinese)

[28] State Administration of Traditional Chinese Medicine. Chung-Hua-Ben-Tsao. Shanghai, China: Shanghai Science Technology Press; 1999. (in Chinese)

[29] Hu, S.Y. 1971. The Orchidaceae of China. Quart. J. Taiwan Mus.24: 67-103.

[30] Chiu, N.Y. and K.H. Chang. 1995. Anoectochilus formosanusHayata, In The Illustrated Medicinal Plants of Taiwan, Vol.4, SMC Publishing Inc., Taipei, Taiwan, pp. 282-283. (inChinese)

[31] Huang, D.D., R.C.S. Law, and O.T. Mak. 1991. Effects of tissue-cultured Anoectochilus formosanus Hay. extracts on the arachidonate metabolism. Bot. Bull. Acad. Sin. 32: 113-119.

[32] Kan, W.S. 1986. Anoectochilus formosanus Hay. In Pharmaceutical Botany, National Research Institute of Chinese Medicine, Taipei, Taiwan, pp. 647. (in Chinese).

[33] Mak, O.T., D.D. Huang, and R.C.S. Law. 1990. Anoectochilus formosanus Hay. contains substances that affect arachidonic acid metabolism. Phytotherapy Res. 4: 45-48.

[34] Lin, J.M., C.C. Lin., H.F. Chiu., J.J. Yang, and S.G. Lee. 1993.Evaluation of the anti-inflammatory and liver-protective effects of Anoectochilus formosanus, Ganoderma lucidum and Gynostemma pentaphyllum in rats. Amer. J. Chin. Med. 21:59-69.

[35] Lin, J.M., C.C. Lin., H.F. Chiu., J.J. Yang, and S.G. Lee. 1993.Evaluation of the anti-inflammatory and liver-protective effects of Anoectochilus formosanus, Ganoderma lucidum and Gynostemma pentaphyllum in rats. Amer. J. Chin. Med. 21:59-69.

[36] Chan, C.C., C.L. Hou, C.H. Chung, and W.T. Liu. 1994. Evaluation of an in vitro virus culture system for anti-virus study of the Chinese herb. J. Food Drug Anal. 2: 123-132. (in Chinese).