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Development of efficient micropropagation protocol for *Andrographis paniculata* through epicotyledonary node

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Abstract

A*ndrographis paniculata* seeds were cultured on a basal medium of half strength MS medium with activated charcoal and 3% sucrose, incubated at 25 ± 2°C and under a light intensity of about 3000 Lux. The seeds were germinated and elongated after 4-5 weeks. The seedlings (epicotyledonary node) were used as explants. The explants (epicotyledonary node) showed swelling at nodal region which was followed by the emergence of shoot buds. Multiple shoot formation was achieved from per-existing meristems of nodal region of explant. The time taken for shoot initiation from explant was independent of growth hormones and nutrient media but the no. of shoots per explants was dependant on the concentration of growth hormones. The epicotyledonary node explants of A*ndrographis paniculata* were inoculated on MS medium containing BAP with IAA and kinetin NAA. The no. of shoots produced per explants varied in different concentration of plant growth regulator but the time period required for shoot induction in all the treatment was similar. As the BAP concentration increased from 2.21µM/lit to 13.31µM/lit the no. of shoot buds were also increased. The maximum number of shoots (20 shoots with 33.5mm length) was observed on BAP (13.31µM/lit) with combination of IAA (5.70µM/lit). As far as the effect of various concentration of BAP, and kinetin with IAA and NAA on shoots regeneration of explants (epicotyledonary node) of A*ndrographis paniculata*, it was concluded that BAP with combination of IAA were suitable for induction of maximum multiple shoots but the concentration of BAP higher than 13.31µM/lit with IAA 5.70 µM/lit, the shoots no. was reduced. On the medium containing kinetin 13.93µM/lit and NAA 4.83µM/lit the growth of shoot bud enhanced. The lower concentration of NAA was ineffective for growth of shoots where as the concentration of NAA higher than 1.07µM/lit inhibited the shoot growth and have induced callusing at the base of shoots. Excised shoots regenerated on MS medium containing BAP 13.31µM/lit and IAA 2.85µM/lit were transferred on to the rooting medium individually. The IBA, NAA and IAA were used individually and in combination to induce root in the regenerated shoots. Although root induction was achieved on auxin free MS medium but these roots were of poor growth. The effect of IBA (4.92µM/lit to 24.60µM/lit) was also studied which revealed that on 19.68µM/lit IBA root induction was 95% within 4-5 weeks on the lower concentration of IBA rooting reduced to 41% and in the concentration of IBA higher than 19.68µM/lit only 90% shoots were rooted with callus. When NAA with combination of IAA in various concentration was used. NAA (10.74µM/lit) and IAA (11.41µM/lit) in MS medium were found to be optimum for achieving maximum (74.8%) rooting in micro shoots of A*ndrographis paniculata.* The rooted plantlets were hardened by keeping them in hardening chamber for 3-4 weeks. After 3-4 weeks of hardening 100 plantlets were transferred to the field conditions where 75 plants could survive.

Keywords: *Andrographis paniculata* media, *in vitro*, seedling segment

List of Abbreviations:
Benzylamino-purine (BAP) Kinetin (KIN) Indole 3-acetic acid (IAA) ά Naphthalene acetic acid (NAA) and 2, 4-Diclorophenoxy acetic acid (2, 4-D)

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1. Introduction

Andrographis paniculata is an erect annual herb and extremely bitter in taste. It has glabrous leaves and white flowers with rose-purple spots on the petals Andrographis paniculata is a promising new herb for the treatment of many diseases including HIV-AIDS and the myriad of symptoms associated with autoimmune disorders. It has been used for medicinal purposes for centuries in India and China. In the west it is sometimes referred to as Indian Echinacea Active Ingredients in *Andrographis paniculata*: *Andrographis paniculata* contains bitter principles andrographolide, a bicyclic diterpenoid lactone and Kalmeghin.Andrographis his paniculata Nees is a slender upright annual varying in height from 30 to 100 cm (1 to 3 feet), with a square stem and "lanceolate" leaves (i.e., shaped like a lance, sharp at the ends and curved in the middle).

Andrographis is indigenous to the plains of India where it is called Kalmegh (in the Bengali language) or Kiryat (in the Hindi language). It is cultivated throughout Southeast Asia and in China where it is called Chuan Xin Lian, and has also been introduced to the West Indies Andrographis does not contain and has nothing to do with "androgens" (testosterone) or any steroids. The active components of andrographis are "andrographolides," very bitter compounds known as diterpene lactones found in the aerial parts of the plant (leaves and stems). The andrographolide content of the leaves varies considerably from season to season, such that standardized extracts are much more reliable as means of supplementation than the leaves or the whole plant. Studies performed in the last 15 years in several locations and published in Western journals have confirmed and adapted many of the traditional uses of andrographis for ailments of our own. The use of andrographis in reducing the incidence or severity of upper respiratory tract infections, including colds and other manifestations, such as tonsillitis and laryngitis, has been popular in Scandinavia over the last decade, and is beginning to gain acceptance in North America. A good review has been published in The Natural Pharmacist. Publications resulting from several clinical studies by researchers in Chile and Sweden provide a credible body of literature. *Andrographis paniculata* reduces the incidence and severity of the common cold. In a randomized, placebo-controlled, double-blind study to assess the possible preventive effect of *Andrographis paniculata* against the common cold, 107 students were randomized to the Andrographis group (n=54, 200 mg Andrographis supplying a minimum of 11 mg of andrographolides, a very low dose) or to the control group (n=53, placebo).

The study was carried out for three months during the winter. The incidence of colds in the Andrographis-treated subjects was 30%, as compared to 62% in the control group (association between Andrographis and absence of colds significant, p[less than] 0.01).In the first of several controlled studies aimed at assessing the effect of Andrographis on symptoms of the common cold, 33 patients received 1,200 mg of *Andrographis paniculata* containing a minimum of 48 mg of andrographolides, while 28 control patients received a placebo. After four days, Andrographis was found to have a significant beneficial effect on tiredness, shivering, sore throat, muscular aches,

rhinitis, sinus pains, and overall disease, but not on lymphatic swelling. Similar results have been obtained in additional studies using the same or similar doses. Altogether, controlled studies involving over 500 subjects indicate that Andrographis paniculata is effective at reducing the prevalence and intensity of colds and sinusitis, and shortening the duration of the symptoms. What is the mechanism of this effect? One possibility is that Andrographis is an immune booster, a possibility supported by the fact that it stimulates several immune parameters in mice. The results suggest that extracts are more potent than purified andrographolides. In addition, research suggests that andrographolides have a direct antiviral effect and a direct antiparasitic effect, but not a direct antibacterial effect. Although the present results are insufficient for a general recommendation for use in viral or parasitic conditions, they open the door for potential future applications.

Coronary Artery Disease and Liver Protection In addition to its usefulness in colds, preliminary research in animals has indicated that Andrographis may be useful in preventing coronary heart disease (CHD), and especially in preventing a condition associated with the treatment of CHD that has been very difficult to control -- restenosis. Restenosis is defined as the rapid return of atherosclerotic blockage following coronary angioplasty, a technique widely used by cardiologists to open up blocked coronary arteries. It is well accepted that fish oil has beneficial effects in the prevention and management of cardiovascular disease. However, even though it may help, fish oil has not conclusively been shown to reduce restenosis following angioplasty. In an animal model, andrographis was shown to be twice as effective as fish oil in preventing the incidence and severity of restenosis following angioplasty. The mechanism may be the antithrombotic effect of andrographis, which may occur as a result of decreases in thromboxane and platelet aggregation.

2. Materials and Method

Preparation of plant materials:

The collected seeds were first washed with a solution of Tween-80 which followed by several washes with distilled water. Surface sterilized seeds of *Andrographis paniculata* were cultured on half strength MS basal medium with activated charcoal (100mg/lit) and 3% sucrose, incubated at 25$\pm$2° C and under 1500-2000 lux intensity of yellow and white light. The seeds were germinated and elongated after 4-5 weeks. The seedlings were used as explants. The explants (epicotyledonary node) showed swelling at nodal region which was followed by the emergence of shoot buds.

Sub-culture and multiplication of propagules:

After establishment of explants aseptically in culture media. The sub-culturing was carried out in some cultures for 5-6 times to multiply them for further experiments. The tissues were subcultured regularly after an interval of 4-5 weeks. The growth hormones were used either individually or in combinations of different concentrations.

Callus Development

Epicotyledonary node explants (length 26.5mm) from sterile *in vitro* seedling were cultured on MS medium containing different concentration of 2,4-D and IAA + NAA for 4 weeks.

Root induction

When the aseptic shoots were raised in sufficient numbers, these were transferred to root inducing media like White's MS basal, MS ½ salt strength ¾ and ¼ strength of MS Salts. *In vitro* produced shoots were also transferred to the other media like B5 basal medium, WP medium and WS medium supplemented with vitamin, amino acids, and various root inducing hormones like NAA, IAA, and IBA. The physical conditions, concentrations and combination of growth regulators, vitamins, amino acids and inorganic salts of media were changed according to the need of culture.

Statistical Design

Experiments were set up in Randomized Block Design (RBD) and 10 replicates for each treatment were tested for shooting medium and 10 replicates for each treatment were tested for *in vitro* regeneration. Data from each experimental stage were analyzed separately and mean and SD (Standard Deviation) of each experimental stage were calculated.

3. Results and Discussion

In vitro* regeneration, multiplication and rooting of *Andrographis paniculata.

A*ndrographis paniculata* seeds were cultured on a basal medium of half strength MS medium with activated charcoal and 3% sucrose, incubated at 25 $\pm$ 2°C and under a light intensity of about 3000 Lux. The seeds were germinated and elongated after 4-5 weeks. The seedlings (epicotyledonary node) were used as explants. The explants (epicotyledonary node) showed swelling at nodal region which was followed by the emergence of shoot buds. Multiple shoot formation was achieved from per-existing meristems of nodal region of explant. The time taken for shoot initiation from explant was independent of growth hormones and nutrient media but the no. of shoots per

explants was dependant on the concentration of growth hormones (Table-1). The epicotyledonary node explants of A*ndrographis paniculata* were inoculated on MS medium containing BAP with IAA and kinetin NAA. The no. of shoots produced per explants varied in different concentration of plant growth regulator but the time period required for shoot induction in all the treatment was similar. As the BAP concentration increased from 2.21µM/lit to 13.31µM/lit the no. of shoot buds were also increased. The maximum number of shoots (20 shoots with 33.5mm length) was observed on BAP (13.31µM/lit) with combination of IAA (5.70µM/lit). As far as the effect of various concentration of BAP, and kinetin with IAA and NAA on shoots regeneration of explants (epicotyledonary node) of A*ndrographis paniculata*, it was concluded that BAP with combination of IAA were suitable for induction of maximum multiple shoots but the concentration of BAP higher than 13.31µM/lit with IAA 5.70 µM/lit, the shoots no. was reduced. On the medium containing kinetin 13.93µM/lit and NAA 4.83µM/lit the growth of shoot bud enhanced. The lower concentration of NAA was ineffective for growth of shoots where as the concentration of NAA higher than 1.07µM/lit inhibited the shoot growth and have induced callusing at the base of shoots. Excised shoots regenerated on MS medium containing BAP 13.31µM/lit and IAA 2.85µM/lit were transferred on to the rooting medium individually.

The IBA, NAA and IAA were used individually and in combination to induce root in the regenerated shoots. Although root induction was achieved on auxin free MS medium but these roots were of poor growth. The effect of IBA (4.92µM/lit to 24.60µM/lit) was also studied which revealed that on 19.68µM/lit IBA root induction was 95% within 4-5 weeks on the lower concentration of IBA rooting reduced to 41% and in the concentration of IBA higher than 19.68µM/lit only 90% shoots were rooted with callus. When NAA with combination of IAA in various concentration was used. NAA (10.74µM/lit) and IAA (11.41µM/lit) in MS medium were found to be optimum for achieving maximum (74.8%) rooting in micro shoots of A*ndrographis paniculata*. The rooted plantlets were hardened by keeping them in hardening chamber for 3-4 weeks. After 3-4 weeks of hardening 100 plantlets were transferred to the field conditions where 75 plants could survive.

In vitro* clonal propagation of Andrographis *paniculata:

The *in vitro* techniques of axillary and terminal buds and stem culture provide opportunity to micro propagate plants this method of vegetative propagation, has advantage over conventional methods e.g. small amount of initial material, rapid multiplication, easy transportation, preservation of germplasm and uniform production throughout the year. The epicotyledonary node, can be stimulated to grow under appropriate cultural conditions (Phyto-hormone type and concentration, light intensity and temperature) subsequently individual shoots are excised for root induction. Experiments were conducted for regeneration of maximum number of shoots from epicotyledonary node explants, using different culture media namely MS medium, supplemented with auxins and cytokinins in various concentration and combinations. The explants showed swelling at nodal region which was followed by the emergence of shoot buds. Multiple shoot formation was achieved from pre-existing meristems of nodal region of explants under high intensity of light (2500-3000 Lux) at 25$\pm$ 2˚C temperature the time taken for shoot initiation from explant was independent of growth hormones and media but the number of shoots were dependent on the kind and concentration of growth hormones.

Effect of BAP and kinetin:

The results obtained are presented that the number of shoots produced per explant varied in various concentration of BAP / kinetin. The shoot number increased from 5 to 15 from such explants as the concentration of BAP was raised from 2.21µM/lit to 22.19µM/lit. The maximum number of shoots (20 shoots with 33.5mm length) was produced on 13.31µM/lit BAP from epicotyledonary node explant. The higher concentration of BAP (22.19µM/lit) inhibited the growth of shoots. The shoot formation was also achieved from epicotyledonary node explants on kinetin supplemented medium. On medium containing kinetin (2.32µM/lit to 18.58µM/lit) only 16 shoots were produced from explant. The higher concentration of kinetin (18.58µM/lit to 23.23µM/lit) in the medium inhibited the shoot proliferation from explants.

Combined effect of BAP+IAA:

The shoot buds regenerated from node of explant, however grew poorly. In order to enhance the growth and vitality of such buds several attempts were made by incorporating different auxins. On BAP and IAA supplemented medium the number of shoots produced was increased on IAA (5.70µM/lit) supplemented with BAP (13.31µM/lit) 20 shoot buds were produced from the explant. As concentration of IAA increased the number of shoot buds decreased and the explant showed callusing. These experiments proved that for induction of healthy multiple shoot buds the explants should be cultured on MS medium supplemented with BAP (13.31µM/lit) and IAA (5.70µM/lit).

Effect of kinetin + NAA:

Results obtained are presented in these prove that the kinetin in combination with NAA was not found as effective as BAP for induction as well as for growth of shoots from subcultured shoot buds. The kinetin (2.32µM/lit to 6.71µM/lit) was incorporated in the medium. On lower concentration of kinetin up to 2.32µM/lit with NAA

0.53µM/lit only 4 shoots (11mm length) were produced. As the concentration of kinetin increased to 13.93µM/lit with increased concentration of NAA 4.83µM/lit the maximum shoots (16 shoots with 25.5mm length) were produced. The length of shoots on higher concentration of kinetin (18.58µM/lit) and NAA (6.71µM/lit) was reduced.

Multiplication of propagules in Andrographis *paniculata:*

Once the proliferation of shoots is achieved from *in vitro* cultured explants. The next step is to multiply the *in vitro* grown shoots. In Andrographis *paniculata* during present investigation experiments were conducted to further multiply the shoot buds regenerated from epicotyledonary node explants. These shoot buds after 4 weeks of culture, when attained a length of 33.5mm were excised from explants and carefully subcultured to the fresh MS medium containing various growth regulators in their combinations were used.

Effect of cytokinins on shoot multiplication:

The shoot buds achieved from epicotyledonary node explants cultured on MS medium containing BAP (13.31µM/lit**) +** IAA (5.70µM/lit) were further subcultured on the MS medium with BAP/ kinetin alone.

Effect of BAP + IAA:

Results obtained are presented in these prove that the BAP in combination with IAA, same concentration for shoot induction and multiplication were used. The no. of shoots (26 shoots with 26.5mm length) and length increased in same medium.

Effect of 2, 4-D and IAA + NAA on callus induction:

Epicotyledonary node explants (length 26.5mm) from sterile *in vitro* seedling were cultured on MS medium containing different concentration of 2,4-D (4.52µM/lit to 18.09µM/lit) and IAA + NAA (IAA 2.85µM/lit to 11.41µM/lit and NAA 2.68µM/lit to 10.74µM/lit) for 4 weeks. The 2,4-D (4.52µM/lit) and NAA + IAA (NAA 2.68µM/lit, IAA 2.85µM/lit) was added in the medium the explants produce 3 shoots with 7mm length and callus induction. As the concentration of 2,4-D increased 18.09µM/lit to 22.62µM/lit the shoot formation was inhibited and callus formation were achieved. On 2,4-D 22.62µM/lit vigorous callus was produced by explants.

Effect of MS + IBA and IAA +NAA on rooting:

Rooting of micro-shoots from the selected treatments was tried on two different media, both containing MS basal medium with 3% sucrose. The regenerated shoots were inoculated in MS + IBA (4.92µM/lit to 19.68µM/lit) combination medium. After 4-5 weeks root induction were produced (41% to 95%) with length of 14.4mm to 36.4mm. The results obtained are presented in Table-3.These reveal that the rooting of micro shoots was influenced by concentration of IBA. On lower concentration of IBA (1.23µM/lit to3.69µM/lit) these shoots did not root and could be rooted only on medium containing 4.92µM/lit to 19.68µM/lit IBA. However, even on these concentrations more time was required (30-40 days) for rooting. Thus for good rooting on IBA 19.68µM/lit and NAA 10.74µM/lit + IAA 11.41µM/lit was optimum for rooting of micro shoots of Andrographis *paniculata*. The roots so produced attained 36.4mm length in 5-6 weeks. The plantlets so developed were however, showed decline in growth.

Table 1. Effect of different Concentration of growth regulators in MS-Medium on regeneration and multiplication of axillary shoots from epicotiledonary node explants of *Andrographis Paniculata.* (Figure-1)

Cytokinins		**Auxins**		**% shoots regeneration (Mean± S.D.)**	**Number of axillary shoots (Mean ±S.D.)**	**Length of shoots (MM) (Mean± S.D.)**
BAP (µM)	**KIN (µM)**	**IAA (µM)**	**NAA (µM)**			
2.21	----	0.57	----	54.5 ± 1.72	5±0.63	12.5 ± 1.39
4.43	----	1.14	----	68.5 ± 0.91	6±0.31	14.0 ± 0.69
8.87	----	4.28	----	76.5 ± 1.41	8±0.56	17.5 ± 0.70
11.09	----	5.70	----	87.5 ± 1.72	11±0.23	19.0 ± 0.97
13.31	----	7.13	----	97.5 ± 1.63	20±1.63	33.5 ± 1.61
17.75	----	9.98	----	92.5 ± 1.20	17±1.07	29.0 ± 1.41
22.19	----	11.41	----	80.5 ± 1.43	15±0.41	27.5 ± 1.29
----	2.32	----	0.53	49.5 ± 1.85	4±0.21	11.0 ± 0.21
----	4.64	----	1.07	53.5 ± 1.22	7±0.32	13.5 ± 0.59
----	6.96	----	1.61	64.5 ± 1.07	8±0.44	16.0 ± 0.80
----	9.29	----	2.68	76.5 ± 0.86	11±0.71	18.5 ± 0.84
----	13.93	----	4.83	95.0 ± 0.92	16±0.91	25.5 ± 1.21
----	18.58	----	6.71	80.5 ± 0.71	12±0.81	22.0 ± 1.01

Each value represents the mean ± SD of 10 replicates

Figure 1. *In vitro* regeneration (shoot initiation and Multiplication

Table 2. Effect of auxins on callus formation in. *Andrographis Paniculata*(Figure 2)

2,4-D (µM)	IAA (µM)	NAA (µM)	Response of shoots for callusing
4.52	----	----	−
6.78	----	----	+
9.04	----	----	+
13.57	----	----	++
18.09	----	----	+++
22.62	----	----	+++
----	2.85	2.68	−
----	5.70	5.37	−
----	11.41	10.74	+
----	17.12	16.11	++

− = No callusing, + = Little callusing,
++ = Moderate callusing,
+++ = Vigorous callusing. (Each value represents the mean ± SD of 10 replicates)

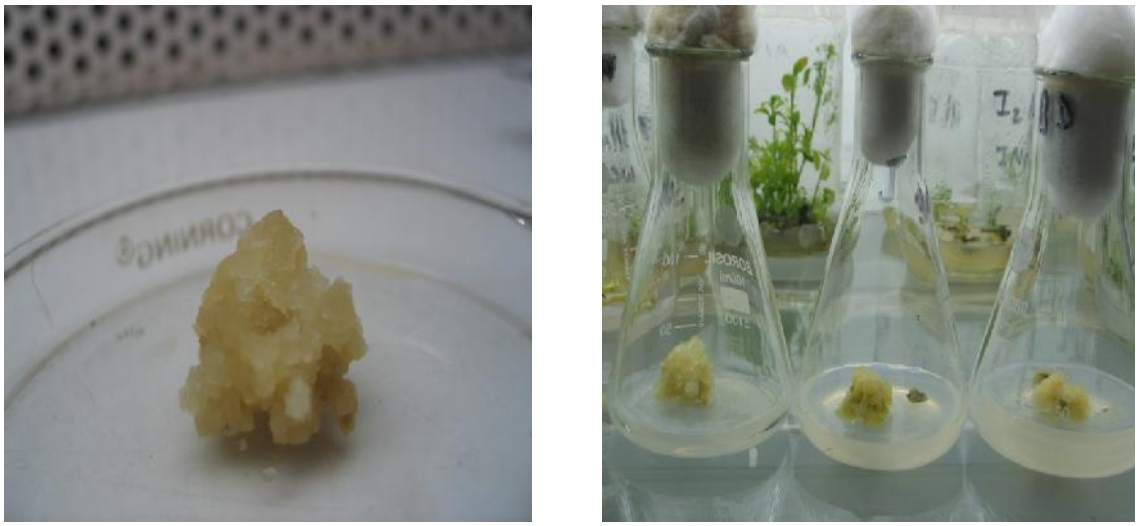

Figure 2. Effect of 2, 4-D and IAA + NAA on callus induction

Table 3. Effect of auxins (IBA, NAA and IAA) on rooting behavior of cultured shoots of *Andrographis Paniculata.* (Figure 3)

Auxins			Percentage of Rooting in micro shoots (Mean ± S.D)	Root Length of (MM) (Mean ± S.D.)	Callusing
IBA (µM)	NAA (µM)	IAA (µM)			
4.92	----	----	41.0 ±1.41	14.4 ± 1.85	−
9.84	----	----	64.6±1.85	18.0 ± 1.41	−
14.76	----	----	84.6±1.85	31.6 ± 1.85	−
19.68	----	----	95.0 ± 2.28	36.4 ± 1.85	−
24.60	----	----	90.8 ± 1.72	32.2 ± 2.13	−
----	2.68	2.85	30.0 ± 1.41	10.5 ± 0.70	−
----	5.37	5.70	45.0 ± 1.41	17.0 ± 0.70	−
----	8.05	8.56	65.0 ± 1.41	19.7 ± 1.07	−
----	10.74	11.41	74.8 ± 2.31	25.8 ± 1.02	+
----	16.11	17.12	60.4 ± 1.85	21.4 ± 1.06	+

− = No callusing, + = Little callusing,
++ = Moderate callusing, +++ = Vigorous callusing. (Each value represents the mean ± SD of 10 replicates)

Figure 3. Effect of MS + IBA and MS + IAA + NAA on rooting

4. Conclusion

Based on current research and financial investments, medicinal plants will, seemingly, continue to play an important role as a health aid. Medicinal plants, since times immemorial, have been used in virtually all cultures as a source of medicine. Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies. Moreover, indeed, the market and public demand has been so great that there is a great risk that many medicinal plants today, face either extinction or loss of genetic diversity. This plant have also been appreciated and recognized for their aesthetic and ornamental value. Such popularity of healthcare plant-derived products has been traced to their increasing acceptance and use in the cosmetic industry as well as to increasing public costs in the daily maintenance of personal health and well being.

Examples of such beauty-oriented therapeuticals are skin tissue regenerators, anti-wrinkling agents and anti-age creams. Most dermaceuticals are derived from algal extracts that are rich in minerals and the vitamin B group. Skincare products such as skin creams, skin tonics, etc. derived from medicinal plants are grouped together as dermaceuticals. Also, amongst the poor, cures and drugs, derived from plants, constitute the main source of healthcare products. Medicinal plants are an integral component of ethno veterinary medicine. Farmers and

pastoralists in several countries use medicinal plants in the maintenance and conservation of the healthcare of livestock. A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional foods, nutriceuticals and nutraceuticals.

To conclude, the successful tissue culture of Andrographis *paniculata* provides a system that is efficient for propagation of this valuable medicinal plant \en masse. It could support conservation and ultimately enable to keep pace with commercial and keep off the species from indiscriminate exploitation from the natural resources. The described protocol could be worked as a useful tool for adapting in vitro culture strategies to increase the biomass of tissue production.

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