EFFECT OF COLCHICINE ON PRODUCTION OF SECONDARY METABOLITES FROM CALLUS OF *WITHANIA SOMNIFERA* (L.) DUNAL

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ABSTRACT

Micropropagation of *Withania somnifera* (L.) Dunal was carried out in MS Media supplemented with various combinations and concentrations of NAA and BAP. Aberrant (Tetraploid) plants of *W. somnifera* (2n=24, 4n≈48) were developed by treating *in vitro* grown shoot tip with 20 mg/l colchicine. Higher concentrations of secondary metabolites (alkaloids, flavonoids and saponin) were found in natural roots than in callus treated with or without colchicine. However the secondary metabolite contents in callus of *W. Somnifera* without colchicine treatment were higher than in that with colchicine treatment.

Key words: Colchicine, *In vitro*, tetraploid, concentration

INTRODUCTION

The importance of medicinal & aromatic plants (MAPs) has increased progressively over the last two decades. Almost 90 % of world population relies on plant-based medicines for primary healthcare. Over 120 compounds from 90 plant species are available as prescription drugs. Asian and European markets dominate worldwide sales of plant based medicinal products. The average value of such markets was put around US$ 20 billion a year (Gruenwald, 1999). Medicinal plants constitute 80 % of raw material for preparation of traditional drugs. They contribute at least 25 % in modern drugs (Rawal and Karki, 2004). As a result, there is growing demand of Nepalese MAPs and other non-timber forestry products (NTFPs). However, the commercial exploitation without any conservation measures has threatened many species. Medicinal and aromatic plant data base of Nepal has listed 1624 species having ethno botanical importance (Shrestha *et al*, 2000).

Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for both studying and producing plant secondary metabolites. Different strategies, using an *in vitro* system, have been extensively studied to improve the production of plant chemicals (Vanisree *et al* 2004).

Withania somnifera is an important medicinal plant. This plant grows widely in all drier part of sub tropical region. It occurs in Congo, South Africa, Egypt, Morocco, Jordan, Pakistan, Afghanistan, Shrilanka, Bangladesh and India. In Nepal it is cultivated for its medicinal value. Several types of alkaloids are found in this plant of which, withanin and somniferine are important in Ayurvedic and Unani preparations. Roots, barks, leaves, fruits and seeds are used to cure various ailments. There are over 20 other species of *Withania* genus that occur in the dry parts of India, North Africa, Middle East and Mediterranean. Only two species *W. somnifera* and *W. coagulant* are reported from Nepal.

In Ayurveda, *W. somnifera* (Ashwagandha) is considered a rasayana herb, which works on a non specific basis to increase health and longevity. This herb is also considered an adaptogen which is a non toxic herb that works on a non-specific basis to normalize physiological function, working on neuro-endocrine system. The traditional use of Ashwagandha was to increase energy, youthful vigor, endurance, strength, health, nature the time element of the body, increase vital fluids, muscles fats, body lymph, and semen cell production. According to Gupta and Rana (2007), withanolides (steroidal lactones with ergostane skeleton) glycowithanolides and alkaloids (withanine is the main constituent, plus semniferine, somnine, somniferinine, withananine, pseudo-withanine, torpine, pseudo-torpine, (delta)-a-glyloxytropan, choline, cuscohygrine) are the major phytochemical constituents of *W. somnifera*. Two acyl steryl glucoside viz sitomtoindoside VII and sitoindiside VIII have been isolated from roots. The leaves contain steroidal lactones, which are commonly called withanolides. The withanolides have C28 steroidal nucleus with C9 side chain, having six membered lactone rings (Gupta and Rana 2007).

Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics. The focus of the present investigation is to quantify the application of tissue culture technology for the production of some important plant pharmaceuticals from *W. Somnifera*. The possibility of *In vitro* cultures and commercial production of some

callus based important secondary metabolites is also explored.

MATERIALS AND METHODS

The materials used for the present investigation were young shoots of *in vitro* grown plants of *Withania somnifera* obtained from Plant Biotechnology Unit, Central Department of Botany, T.U, Kirtipur, Kathmandu.

Murasinge and Skoog (1962) MS basal was used as the basal medium for the micropropagation and mass scale callus production. Two types of plant growth regulators Auxins (α naphthalene acetic acid -NAA) and Cytokinins (6-Benzylaminopurine- BAP) were supplemented either alone or in combination in the media.

On agar medium, 20 mg/l colchine was supplemented to induce the ploidy in the *in vitro* regenerated plants or callus. After inducing polyploidy, the combination of NAA (0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2.0 mg/l) and BAP (0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2.0 mg/l) was used to investigate the effect of hormone on callus induction and proliferation, so produced callus was used for phytochemical test.

The natural roots of *W. somnifera* were collected from cultivated field from Chitwan, Nepal and ground to the fine powder. After 8 weeks of culture, the callus was harvested, air dried, grounded and the powder was used for the qualitative and quantitative analysis of different secondary metabolites.

Alkaloids, tannins, saponins, steroid, terpenoid, flavonoids, phlobatannin and cardic glycosides content were determined by using the methods of Edeoga *et al.* (2005).

Qualitative test for different secondary metabolites

Test for tannins:

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added.

Test for phlobatannins:

Aqueous fraction was extracted soaking the powdered sample for 48 hours in distilled water and filtering the solution. The aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid

Test for saponin:

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed.

Test for flavonoids:

Aqueous fraction was extracted soaking the powdered sample for 48 hours and filtering the solution. 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of conc. H_2S0_4. A yellow colouration observed in each extract. The yellow colouration disappeared on standing. Few drops of 1% aluminium solution were added to a portion of each filtrate.

Test for terpenoids:

Aqueous extract and ethanol fraction were extracted soaking the powdered sample in distilled water and 70% ethanol for 48 hours and filtering the solution Five ml of each (aqueous and ethanol) extract was mixed in 2 ml of chloroform, and 3 ml of concentrated H_2S0_4 was added carefully.

Test for cardiac glycosides (Keller-Killani test)

Aqueous extract and ethanol fraction were extracted soaking the powdered sample in distilled water and 70% ethanol for 48 hours and filtering the solution. Five ml of each extracts (aqueous and ethanol) was treated with 2 ml of glacial acetic acid containing one drop oetf ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid.

Quantitative determination of the chemical constituents

Alkaloid determination using Harborne (1973) method:

Five gm of the sample were weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a waterbath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Saponin determination:

The samples were ground and 20 g of each were put into a conical flask and 100 ml of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml

of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight; the saponin content was calculated as percentage.

Flavonoid determination:

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

2.2.11 Statistical analysis

Means were calculated from three replicates. Statistical analysis was done by SPSS version 16.0. Data were analyzed using correlation coefficient and means were compared using Independent-Samples T-Test with a significant level of ≤ 0.05.

RESULTS AND DISCUSSION

Qualitative test

The natural roots as well as the callus (both colchicines treated and untreated) of *W. somnifera* have been found to contain different secondary metabolites such as alkaloids, flavonoid, saponin, phenolic compounds, tanin, phlobatanins, terpenoids and cardiac glycosides. The result shows distinct presence of alkaloids, flavonoid, saponin, phenolic compounds, tanin and slight notification of presence of phlobatanins, terpenoids and cardiac glycosides.

Quantitative Phytochemical Taste

Some of the secondary metabolites (alkaloids, Flavonoids and Saponins) in callus developed

Table-1: Qualitative test for different secondary metabolites

Plant Materials	Alkaloid	Flavonoids	Saponin	Phenols	Tanin	Phlobatannins	Terpenoids	Cardiac glycosides
Natural Roots	+++	+++	+++	+++	+++	++	++	+
Callus	+++	+++	+++	+++	+++	+	+	+
Callus treated with colchicine	+++	+++	+++	+++	+++	++	++	+

Note: + positive notification of presence of the tested group, + ++ Distinct notification, ++ Clear notification, - Absence of the group tested

Table-2: Quantitative alkaloid, flavonoid and saponin content (%);

	Natural roots (R)	Callus (C)	Callus with colchicine (C)	t- value^{sig}		
				(R vs C)	(R vs CC)	(C vs CC)
Alkaloid	1.32±.73	1.01±.18	0.85±.08	**0.72$^{.052}$**	**1.11$^{.029}$**	1.31$^{.198}$
Flavonoid	2.030±.24	1.70±.20	1.33±.32	1.837$^{0.75}$	3.06$^{0.55}$	1.69$^{0.39}$
Saponin	1.88±.34	1.49±.33	1.77±.66	1.436$^{0.896}$	0.250$^{0.176}$	-0.673$^{0.168}$

± SD, **Note:** Bold figures are statistically significant at 5% level of significance

from colchicine treated shoot tips contained higher concentration as compared to the secondary metabolites in non treated callus, though statistically the difference was not significant. The secondary metabolites in callus with or without colchicines are less in concentration than in the natural roots, though the mean difference is not significant statistically (Tab 2). Gao et. al (1996) reported colchicines treated *Salvia miltiorrhiza* with bigger size and higher amount of chemical compound as compared to the controlled.

The natural roots as well as the callus contained Alkaloids, Flavonoid, Saponin, Phenolic compounds, Tanin, Phlobatanins, Terpenoids and Cardiac glycosides. The average Alkaloid (1.32 %, 1.007 % & 0.820), Flavonoid (2.03 %, 1.7 % & 1.333 %) and Saponin (1.88 %, 1.487 % & 1.773 %) were found in natural roots, callus and callus treated with colchicine of *W. somnifera* respectively. The colchicine treated callus of Ashogandha shows lesser content of secondary metabolites than the untreated callus. However the phenotype of colchicine treated plantlet was more vigorous than the colchicine not treated plantlet. The result so obtained is similar as reported by Kim *et al.* (2004) in *Panax ginseng*.

The independent sample t-test in Table 2 shows that the mean difference between the alkaloid content of natural roots and callus as well as callus treated with 20 mg/l colchicines were significantly different. There was no significant mean difference in alkaloid content in both the treated and not treated callus. On mean percentage basis, natural roots were seemed to be rich in flavonoids). Calli of *W. somnifera* are found to be rich in flavonoid content. It was found that colchicine played inhibitory effect in synthesis of flavonoid content. Saponin was highest in natural roots. However, no significant difference on saponin content in natural roots and callus was found.

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