IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 4 Ver. V (Jul -Aug. 2014), PP 01-06
www.iosrjournals.org

Callusing, Cell suspension culture and Secondary Metabolites Production in *Argyreia nervosa* (Burm. F.) Bojer

Priyanka Desai and M.N.Reddy
Shree Bapalal Vaidya Botanical Research Centre, Department of Biosciences, Veer Narmad South Gujarat University, Surat - 395007, Gujarat, India.

Abstract: *In vitro cultures of Argyreia nervosa (*Hawaiian Baby Woodrose) *was initiated form leaf of the plant on Murashige and Skoog's (MS) medium containing different PGRs. Callus induction was experimented by culturing leaf at different combinations and concentrations of PGRs. Best callus induction and fresh weight were obtained at 5.0 mg/l IAA+ 1.0 mg/l BAP. The callus maintained up to 2-3 months. Maximum callus induction and proliferation was noted at 5.0 mg/l IAA + 1.0 mg/l BAP. 2, 4-D gave less callus growth and smaller diameter than IAA and NAA. The callus tissues specifically obtained from leaf and stem explants dispersed in full strength liquid MS medium fortified with 3% sucrose and plant growth regulator combination of IAA (5 mg/l) and BAP (1.0 mg/l) was found best for establishing suspension culture for secondary metabolite production under in vitro condition. Increased sugar level gave better result in cell production and secondary metabolites production. Callus and cells produced equal or higher yield than original plants.*

Keywords: *Argyreia nervosa, callus, cell suspension culture, secondary metabolites.*

I. Introduction

Argyreia nervosa is clambering vine belonging to family Convolvulaceae. It is native to India, from Assam to Belgaum and Mysore. It is common on the Bengal plain (Hooker, 1885). It is found in India throughout up to an altitude of 300 meters high, except in dry western regions. It prefers tropical and sub-tropical climates. The plant prefers fertile, moist soil in a protected sunny position (Ellison, 1995). It grows well in Hawaii, California, Florida and similar climates. The leaves are applied over skin diseases and wounds. Dried leaves are used for diabetes (Jain and Sharma, 1967). Traditionally leaves used by Rajasthani tribes to prevent conception (Anonymous, 1988; Niteswar, 1988).The roots are used in the treatment of gonorrhea, rheumatism and diseases of nervous system. It is also used in obesity, hoarseness, syphilis, anemia, tuberculosis and general debility. It is also used as a tonic (Prajapati et al, 2003).

LSD is the best-known synthetic hallucinogen and is psychoactive at the microgram level. Although LSD does not occur in nature, a close analogue, lysergic acid amide (LSA, ''ergine'') is found in the seeds of Argyreia nervosa. Five to ten seeds of Argyreia nervosa yield average doses of LSA (Al Assmar, 1999; Borsutzky et al., 2002).

Hawaiian Baby Woodrose seeds were traditionally used in sacramental rituals of the Hawaiian and Polynesian islands. Traditional use of the plant in India usually employed the leaves and roots of the plants, which are not psychoactive, as antiseptic and anti-inflammatory drugs. The whole plant is reported to have antiseptic properties.

The petroleum ether extract of the leaves yielded 1-tricontanol, epifriedelinol acetate, epifriedelinol and β-sitosterol (Sahu and Chakravarti, 1971). The leaves were found rich in quercetin (Daniel, 1989). Extraction of the leaves with 90% methanol led to the isolation of the flavonoids, quercetin and kaemperol together with the latter's glycoside kaemperol-3-o-l-rhamnopyranoside (Khan et al., 1992). Two new flavone glycosides characterized as 7,8,3',4',5'-pentahydroxyflavone5-o-α-lrhamnopyranoside and 7,8,3',4',5'-pentahydroxyflavone5-o-α-l-glucopyranoside were also reported from leaves (Ahmad et al., 1993). The hexane extract of the root yielded tetradecanyl palminate, 5, 8-oxidotetracosan-10-one (Rani and Shukla, 1997) and two novel aryl esters characterized as stigmasteryl p-hydroxycinnamate and hexadecanyl phydroxycinnmate along with scopoletin (Shrivastava and Shukla, 1998). The seeds yielded fatty oil which found to contain the glycerides of palminate, stearic, linoleic, linolenic and oleic acids (Biswas et al., 1947; Batra and Mehta 1985). In another study, the seed oil revealed the presence of myristoleic, myristic, palmitic, linoleic, linolenic, oleic, stearic, nonadecanoic, eicosenoic,eicosanoic, heneicosaoic and behenic acids identified as their corresponding methyl esters through GLC (Kelkar et al., 1947). The ethanolic extract of the seeds revealed the presence of a mixture of three alkaloids, out of which only one was characterized as ergometrin. The other constituents isolated were caffeic acid and ethyl caffeate (Agrawal and Rastogi, 1974), another study also revealed the presence of ergoline alkaloids in the seeds (Nair et al., 1987). The ergolines were indicated to be of clavine type (Nair et al., 1987). The free amino acids reported in the seeds were glutamic acid, glycine, isoleucine, leu-cine, lysine, phenylalanine, tyrosine, praline and α-amino butyric acid (Jaiswal et al., 1984). The fruits were reported

to contain n-tricontanol, β-sitosterol, p-hydroxycinnamoyloctadecanolate and caffeic acid (Purushothaman et al., 1982).

II. Materials and methods

Callus:

The plant material was collected from Bapalal Vaidya Botanical Research Centre, VNSGU, Surat, Gujarat, India. The leaf explants were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles from the surface. The explants were then washed with 1.0% liquid detergent (teepol) for another 15 minutes and then washed properly to remove the detergent. The explants were then treated with bavistin (fungicide) for another 10-15 minutes to remove the fungus and then washed properly to remove the fungicide. Primary washing of the explants was done with tap water and DDH_2O for all the explants. The explants then treated with 70% ethanol for 1-2 minutes. The explants treated with 8% Hydrogen peroxide solutions for 5-10 minutes respectively depending upon the explants. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove the traces of Hydrogen peroxide. Fresh cuts were given to the explants after sterilization to remove undesirable or dead portions. The explants were then transferred on MS medium with various growth regulators viz., IAA, NAA, 2, 4-D, BAP, KN and GA_3 in different concentrations and combinations from 0.20 mg/l to 20.00 mg/l for all experiments. 2.5-3% sucrose was added before adjusting the pH between 5.7-5.75 and the media were gelled with 0.7 %(w/v) agar. 20-30 ml media were dispersed in culture tubes and 30-40 ml media in culture jars. These tubes and jars with medium containing growth regulators were sterilized by autoclaving at 121°C and 15 lbs/sq inch pressure for 20-25 minutes. The cultures were incubated at 25±2°C under 24 hours (light/dark) photoperiod with white fluorescent light intensity of 1800-2000 lux. 10 replicates were used for each treatment.

Sub-culturing was carried out at an interval of 30-40 days. The cultures were observed and examined in every day and data was recorded. The resulting callus of all the explants was maintained by frequent subcultured at every 8 weeks in culture tubes containing fresh medium.

The initiation of callus first started from the cut margin of explant ultimately unorganized mass proliferates establishing a callus culture. After every 8 weeks of culture initiation the primary cultures thus obtained having undifferentiated mass of callus were sectioned into small pieces and then transferred to fresh medium for proliferation or regeneration under air laminar flow cabinet. The callus was harvested regularly at the transfer age of 8-9 weeks for analysis.

Cell Suspension Culture:

The media contained MS (Murashige and Skoog, 1962) basal mineral nutrients plus, vitamin and sucrose (3.0-4.0%) as the carbon source. Plant Growth Regulators were added. The pH of the medium was adjusted 5.75 with the help of 0.1 N NaOH and 0.1 N HCl. in each 250 ml flask 50 ml medium was dispensed. All the flasks were plugged with non-absorbent cotton plugs and mouth of tube was wrapped with aluminum foil. These flasks with medium containing growth regulators were sterilized by autoclaving at 121°C and 15 lbs/sq inch pressure for 20 - 25 minutes.

The media were supplemented with BAP and IAA in different concentrations from 1.0 mg/l to 7.00 mg/l has been used for all experiments. Liquid MS Basal sterilized medium without agar was used for cell suspension culture. 50 ml MS medium containing 3% sucrose with respective growth regulators was placed in 250 ml flask. 1 g friable callus was used for cell suspension culture. Cell Piece of callus transferred to a sterilized pertidish in laminar air flow. Gently broke the callus of ~1cm diameter with forceps into 10-20 small pieces. These small pieces of callus were transferred to the liquid media containing different concentration of IAA and BAP (1.0 BAP+1.0 IAA; 1.0 BAP+3.0 IAA; 1.0 BAP+5.0 IAA; 1.0 BAP+7.0 IAA). Opening of the flask was flame sterilized and place the sterilized cap on the flask. Replicated samples were prepared. Sucrose level was increased to check cell growth.

The cultures incubated on an incubating shaker at 120 rpm and 25±2°C. After 10days 5ml liquid media with single cells and small cell clumps was transferred to other flaks containing 50 ml media.

The growth of cells was measure by haemocytometer. 1ml media pipette out from each flask containing different concentration of IAA and BAP in sterile condition and transferred to both chambers of a haemocytometer and a cover-slip was used to allow each chamber to be filled with capillary action.

Cell counting started with one chamber of the haemocytometer, all the cells in the four 1-mm corner squares are counted in each chamber. Each 1-mm square of the haemocytometer represented total volume of $10^{-4}cm^3$. Since cm^3 is equivalent to 1ml, the cell concentration per ml determined as followed:

Cells per ml= the average count per square X dilution factor X 10^4

Total cells= cells per ml X original volume of culture medium from which cell suspension was taken.

1ml media pipette out from flasks at short intervals of time (3-5 days) and counted the cell numbers. Plotted the cell count data of a passage on a graph and the curve indicated the growth pattern of suspension culture.

Evans blue stain was used to check cell viability. Evans blue stain is excluded by living, functional membranes. Thus it is taken up by dead cells and excluded by living cells. This is easily seen under the light microscope.

III. Results:

Argyreia nervosa is a woody climber and very hairy plant. Removal of contaminant from explants was very hard task. Many sterilizing agents like 1-2% Sodium hypochlorite (NaOCl), 0.1-0.2% Mercuric chloride ($HgCl_2$) and 8% Hydrogen peroxide (H_2O_2) were used. In our present work we observed, 8% Hydrogen peroxide (H_2O_2) removed maximum contaminants than other sterilizing agents used during tissue culture work. Contamination rate was highest during monsoon. The explants of the *Argyreia nervosa* showed wide range of variation with the changes in the concentrations and combinations of the PGRs in the media.
Details of the various observations during investigation are described below.

Callus induction:

Culture medium supplemented with different concentrations and combinations of auxins (IAA/NAA/2, 4-D) and cytokinins (BAP/KN) was found to initiate callus formation from leaves, internodes and nodal segments.
All explants showed best response for callus induction.

Callus was initiated from the leaf explants within 7-10 days of inoculation in culture media supplemented with IAA and BAP. At the time of initiation the calli is whitish green in colour but it turned in to pale green in colour after 10 -12 days. Maximum callus induction and proliferation was noted at 5.0 mg/l IAA + 1.0 mg/l BAP with a frequency of (86.66±3.33%) and callus weight was (9.91±0.50 g) after 60 days of inoculation (Table No. 1). This combination was found best among all combination used during investigation. The callus maintained up to 2-3 months.

On media, supplemented with NAA and BA, the leaf explants produce callus. The best combination for callus induction and proliferation was noted at 5.0 mg/l NAA + 1.0 mg/l BAP with a frequency of (66.66±3.33%) and callus weight was (6.45±0.24 g) after 60 days of inoculation (Table No.2). Callus formation started after 10-12 days. The process of callus formation was slow then combination of IAA and BAP.

The role of 2, 4-D in callus induction was the least marked among all used auxins. Callus initiated from explants after 15-20 days and in some cases after 20-25 days. Highest percentage (50.00±5.77%) of explants showing response in the combination was noted at 10.00 mg 2, 4-D + 1.0 mg BAP and weight of callus was (5.68±0.51 g) after 60 days of inoculation (Table No. 3). increase in the concentration of the hormones did not improve the callus growth.

Table No.1
Effect of IAA alone or in different concentration and combination of BAP/KN on callus induction of leaf explants of *Argyreia nervosa* after 4 weeks of inoculation

Growth regulators (mg/l)	Days to initiate callus	Mean % of callus induction ±S.E.	Colour	Texture	Weight of callus (g) after 60 days of inoculation
0.0	12	40.00±5.77	WG	Friable	1.00±0.11
1.0 IAA + 0	15	43.33±3.33	WG	Friable	1.39±0.10
2.0 IAA + 0	13	46.66±3.33	WG	Friable	2.10±0.11
3.0 IAA + 0	11	50.00±0.00	PG	Friable	2.30±0.05
4.0 IAA + 0	10	53.33±3.33	PG	Friable	4.94±0.43
5.0 IAA + 0	9	56.66±5.77	PG	Friable	6.46±0.21
5.0 IAA + 0.5 BAP	8	73.33±3.33	PG	Friable	9.00±0.57
1.0 IAA + 1.0 BAP	13	46.66±3.33	PG	Friable	1.99±0.07
2.0 IAA + 1.0 BAP	11	53.33±3.33	PG	Friable	3.20±0.37
3.0 IAA + 1.0 BAP	10	53.33±3.33	PG	Friable	5.02±0.31
4.0 IAA + 1.0 BAP	9	63.33±3.33	PG	Friable	6.96±0.11
5.0 IAA + 1.0 BAP	7	86.66±3.33	PG	Friable	9.91±0.50
6.0 IAA + 1.0 BAP	7	83.33±3.33	PG	Friable	9.50±0.28

7.0 IAA + 1.0 BAP	9	83.33±3.33	PG	Friable	9.30±0.35
0.2 IAA + 2.0 BAP	14	53.33±3.33	G	Compact	3.23±0.17
0.3 IAA + 3.0 BAP	12	56.66±3.33	G	Compact	3.90±0.15
1.0 IAA + 5.0 BAP	13	50.00±0.00	G	Compact	5.46±0.14
1.0 IAA + 2.0 KN	13	56.66±3.33	G	Compact	3.97±0.13
1.0 IAA + 4.0 KN	11	43.33±11.54	G	Compact	5.50±0.20
1.0 IAA + 6.0 KN	11	50.00±0.00	G	Friable-Compact	6.70±0.43
1.0 IAA + 8.0 KN	10	56.66±05.77	G	Friable-Compact	7.30±0.36
1.0 IAA + 10.0 KN	10	63.33±3.33	G	Friable-Compact	8.00±0.51

Table No.2
Effect of combination of NAA and BAP on callus induction of leaf explants of *Argyreia nervosa* after 4 week of inoculation

Growth regulators (mg/l)	Days to initiate callus	Mean % of callus induction ±S.D.	Colour	Texture	Weight of callus (g) after 60 days of inoculation
1.0 NAA + 0.1 BAP	17	30.00±5.77	PG	Friable	1.91±0.10
2.0 NAA + 0.2 BAP	15	43.33±3.33	PG	Friable	2.76±0.22
3.0 NAA + 0.3 BAP	13	46.66±3.33	PG	Friable	3.61±0.24
4.0 NAA + 0.4 BAP	12	46.66±3.33	PG	Friable	4.53±0.50
5.0 NAA + 0.5 BAP	10	50.00±5.77	PG	Friable	5.81±0.26
5.0 NAA + 1.0 BAP	10	66.66±3.33	PG	Friable	6.45±0.24

Table No. 3
Effect of different combination of 2, 4-D and BAP on callus induction of leaf explants of *Argyreia nervosa* after 4 weeks of inoculation

Growth regulators (mg/l)	Days to initiate callus	Mean % of callus induction ±S.D.	Colour	Texture	Weight of callus (g) after 60 days of inoculation
1.0 2,4-D + 1.0 BAP	20-28	6.66±3.33	PG	Friable	0.82±0.12
2.0 2,4-D + 1.0 BAP	20-25	10.00±5.77	PG	Friable	0.98±0.15
3.0 2,4-D + 1.0 BAP	20-25	13.33±3.33	PG	Friable	1.52±0.16
4.0 2,4-D + 1.0 BAP	17-20	20.00±5.77	PG	Friable	2.50±0.24
5.0 2,4-D + 1.0 BAP	15-18	23.33±3.33	PG	Friable	2.56±0.27
7.0 2,4-D + 1.0 BAP	15-18	33.33±3.33	PG	Friable	3.69±0.29
9.0 2,4-D + 1.0 BAP	18-20	36.66±3.33	PG	Friable	5.17±0.09
10.0 2,4-D + 1.0 BAP	20-25	50.00±5.77	PG	Compact	5.68±0.51

WG= Whitish Green: PG=Pale Green: G=Green
Value represent means ± standard error of 10 replicates per treatment in three repeated experiments

IV. Cell Suspension culture

The growth of cells in the in the suspension culture of leaf during the course of the experiment peaked on the 7th day (Fig.1). The data on packed cell volume (Table No.4) revealed that it increased maximum during the period between 7- 22 days of culturing.

The callus tissues specifically obtained from leaf explants dispersed in full strength liquid MS medium fortified with 3% sucrose and plant growth regulator combination of IAA (5 mg/l) and BAP (1.0 mg/l) was found best for establishing suspension culture for secondary metabolite production under in vitro condition. Increased sugar level gave better result in cell production and secondary metabolites production.

Based on the behaviour of cells in suspension, the early stationary phase was identified to be 10-15 days for leaf suspension culture.

4% sucrose used with same combination for leaf cell suspension culture. Increased sucrose level gave better results in leaf (Table No. 5; Fig.2). Cell numbers increased in leaf suspension culture.

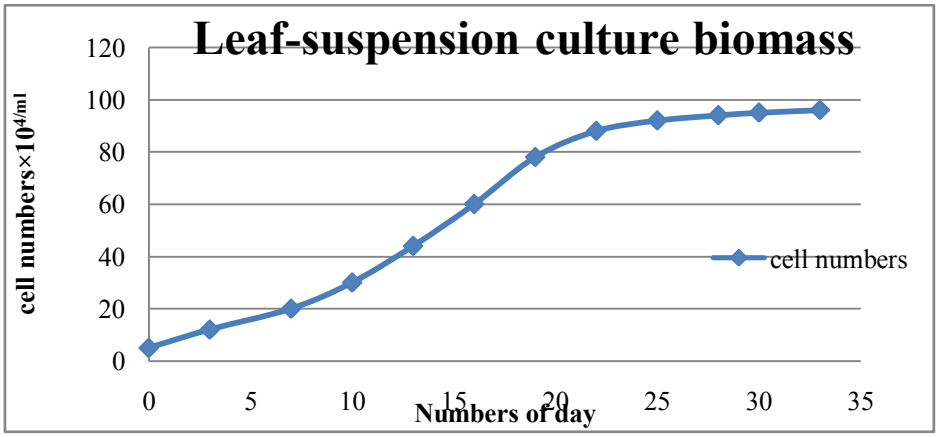

Fig. No.1 Leaf-suspension culture biomass

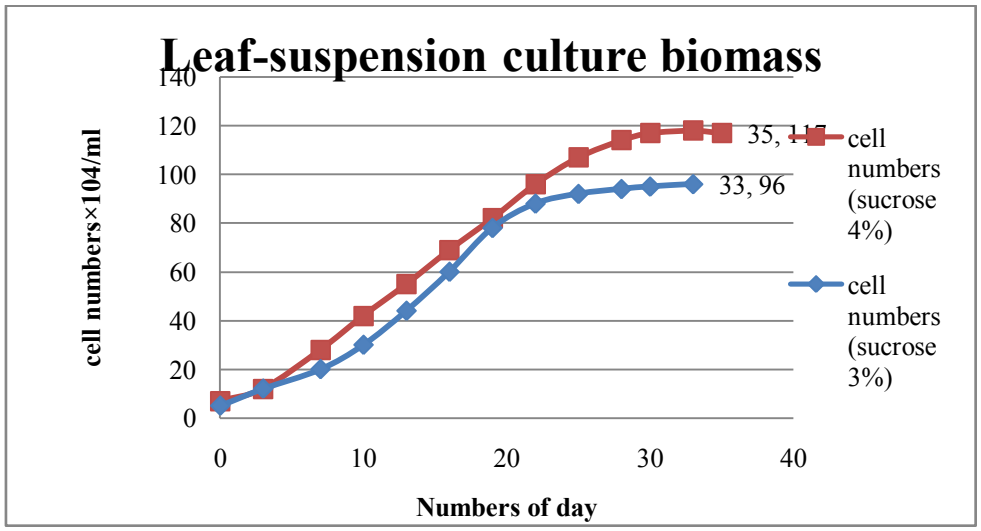

Fig. No. 2 growth of cells in the suspension culture of leaf with increased sucrose (4%) level

Table No. 4 Growth analysis of leaf cell suspension culture

Days	Concentration BAP+IAA(mg/l)				Cell numbers X 10^4 Per ml
	1.0+1.0	1.0+3.0	1.0+5.0	1.0+7.0	
0	7	4	5	6	
3	7	5	12	11	
7	10	9	20	18	
10	13	15	30	27	
13	17	23	44	38	
16	23	31	60	50	
19	30	38	78	62	
22	35	45	88	74	
25	39	49	92	78	
28	42	51	94	83	
30	43	52	95	85	
33	42	53	96	87	
35	42	52	96	88	

Table No. 5
Growth analysis of leaf cell suspension culture with increased sucrose (4%) level

Days	Concentration 1.0 BAP+5.0 IAA(mg/l)	
	Leaf	
0	7	**Cell numbers X 10^4 Per ml**
3	12	
7	28	
10	42	
13	55	
18	69	
21	82	
24	96	
27	107	
30	114	
33	117	
36	118	
40	117	

References

[1]. Agrawal S K and Rastogi R P (1974a): Ergometrine and other constituents of *Argyreia speciosa* sweet. Ind. J. Pharmacol. 36: 118-119.
[2]. Agrawal S R and Rastogi R P (1974b): Pharmacognostical and preliminary phytochemical studies of *Argyreia nervosa* Burm. Indian J. Pharmacol. 35: 118-119.
[3]. Ahmad M, Jain N, Khan M S, Shafiullah, Iliad M (1993): Two new flavone glycosides from the leaves of *Argyreia speciosa*. J. Chem. Res. (S). 7: 248.
[4]. Al Assmar S E (1999): The seeds of the Hawaiian baby woodrose are a powerful hallucinogen. Arch Intern Med. 159: 2090.
[5]. Anonymous (1988): The Welth of India: Raw material, CSIR, 1: 87-88.
[6]. Batra A and Mehta B K (1985): Chomatographic analysis and antibacterial activity of the seed oil of *Argyreia speciosa*. Fitoterapia, 56: 357-359.
[7]. Biswas B, Tiwari L D and Dutt S (1947): Chemical composition of the fixed oil from the seeds of *Argyreia speciosa*. Ind. Soap J., 13: 51-54.
[8]. Borsutzky M, Passie T, Paetzold W, Emrich H M and Schneider U (2002): Hawaiian baby woodrose: (Psycho-) pharmacological effects of the seeds of *Argyreia nervosa*. A case-orientated demonstration, Nervenarzt., 73: 892– 896.
[9]. Daniel M (1989): Polyphenols of some Indian vegetables. Curr. Sci. 58: 1332-1334.
[10]. Ellison D (1995): Cultivated plants of the world, Flora Publications International, Brisbane, pp. 44.
[11]. Hooker J D (1885): The flora of British India, Secretary of State for India Council, London, 4: 185.
[12]. Jain S R and Sharma S N (1967): Hypoglycamic Drugs of India Indigenous Origin. Planta Med, 15 (4): 439-442.
[13]. Jaiswal S, Batra A, Verma S, Bokadia M M (1984): Free amino acids of some regionally available medicinally important plant seeds. Sci. Cult. 50: 24-26.
[14]. Kelkar G M, Phalnikar N L, Bhinde B V (1947): Fatty oil from the seeds of *Argyreia speciosa* Sweet. J. Indian Chem. Soc., 24: 83-86.
[15]. Khan M S, Kamil S M and Ilyas M (1992): Phytochemical investigation on the leaves of *Argyreia speciosa*. J. Indian Chem. Soc., 69: 110-113.
[16]. Nair G G, Daniel M and Sabnis S D (1987): Ergolines in the seeds of some Indian convolvulaceae. Indian J. Pharm. Sci. 49: 100-102.
[17]. Nisteswar K (1988): Review of certain indigenous Antifertility agents, Deerghyu international Govt. Ayurvedic college vijyawada AP India. 4(1): 4-7.
[18]. Prajapati N D, Purohit S S, Sharma A K and Kumar T (2003): A handbook of medicinal plants-complete source book. ISBN: 81-7754-134-X. U500126.
[19]. Purushothaman K K, Arvada A and Loganathan D (1982): Phytochemical study of *Argyreia speciosa* (Vridhadaru). Buli Med. Ethnobot. Res., 3: 250-253.
[20]. Rani A and Shukla Y N (1997): Disubstituted tetrahydrofuran and an ester from *Argyreia speciosa*. Indian J. Chem. 36B: 299-300.
[21]. Sahu N P and Chakravarti R N (1971): Constituents of the leaves of *Argyreia speciosa*. Phytochem. 10: 19-49.
[22]. Shrivastava A and Shukla Y N (1998): Aryl esters and a coumarin from *Argyreia speciosa*. Indian J. Chem., 37B: 192-194.