International Journal of Engineering & Technology, 7 [2.29] [2018] 274-278

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper

Production of reserpine of rauwolfia serpentina [L] kurz ex benth through in vitro culture enriched with plant growth regulators of NAA and kinetin

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Abstract

Rauwolfia serpentine [L] Kurz ex Benth is a valuable plant yielding alkaloid of reserpine. The aim of this study was to find the most effective way of reserpine production. Growth regulators {naphthalene acetic acid [NAA] and Kinetin}were applied to promote and to regulate the growth of explant of R. serpentine cultured in vitro. The study was conducted at the laboratory of Bandung Institute of Technology [ITB]. Nine formulations of NAA and Kinetin were the treatments that were repeated twice in completely randomized design. Weight of callus was measured at one, two, three and four weeks after induction of explant. Reserpine analysis was performed by High Performance Liquid Chromatography [HPLC]. The result showed that the best callus induction was the treatment of 2.5 ppm NAA + 2.5 ppm Kinetin, and the highest content of reserpine was in root organ [0.0021 g / L], and in callus [0.0021 g / L] at the age of 4 weeks after induction. There was revealed that in vitro culture method was more productive in producing reserpine compound than the conventional plantation of R serpentine. Production of reserpine by callus culture was more effective and may be the basic for recommended further effort.

Keywords: *alkaloid; callus; content; effective.*

1. Introduction

Rauwolfia serpentine [L.]Kurz ex Benth is one of the most useful species of more than 100 species of the genus *Rauwolfia*. It has potential benefit as a medicinal plant. *R. serpentine* plant belongs to the family Apocynaceae and plant height may reach 60 cm to 90 cm. *R. Serpentine* plant is native to tropical and subtropical regions of the world, including Europe, Africa, Asia, Australia, and the Central and South America. And is native to moist, deciduous forest of Southeast Asia, including Burma, India, Bangladesh, Sri Lanka, and Malaysia, and Indonesia [1][2]

In India root of the plant is used as an antidote stings, insect anti bites, poison for reptiles and a natural insecticide. In addition to human health, it functions as febrifuge, stimulant for the contraction of urine as well as the treatment of dysentery, insomnia and insanity. The chemical composition in *R. serpentina* has been studied since 1931. More than 50 alkaloids have been isolated and identified [3]

Reserpine is the main component of serpentine which accumulated in roots and is known to have antihypertensive properties. The active compounds in the root of *R serpentine* can lower the high blood pressure than other alkaloids. Research using a root extract of *R. serpentine* showed a good effect in dropping in blood pressure [4]

Studies to obtain reserpine out of the plant as a natural insecticide have been carried out by many researchers. Natural insecticide is considered safer for the environment because it is easily degradable in soil as well as relatively less toxic to the parasitoid [5].

Reserpine of *R. serpentine* can inhibit the feeding process of plant pests [6]. Result of a study showed that reserpine effect on the metabolism of serotonin in the brain ganglia of *Inachisio* [Lepidoptera] [7].

Demand of reserpine in Indonesia is increasing, but the supply of materials of this compound depending on availability of the plant growing in nature because this plant is rarely cultivated that the plant is now rarely found in nature [8]. Therefore, it is a need to cultivate the plant in order to produce up to commercial scale to meet the market demand.

Culture techniques to improve the yield of the plant have been developed among other is the in vitro culture. In tissue culture technique plantlet is able to differentiate and to develop into a complete plant [9]. It is worthwhile to cultivate the plant to fulfill the increasing demand of reserpine and the promising option in the pharmaceutical world due to the presence of the significant chemical compounds in roots. [10]

A study showed positive effect of Kinetin and NAA on callus induction and root formation in vitro grown with leaf explants if we use suitable concentrations of them, alone or in combination [11]; [12]

NAA as growth regulator generates the growth of root, and maximum shoots grew from callus of explant inoculated in medium containing BAP [2.0 mg/L] plus NAA [0.5 mg /L] [1]. Reported that explant of *R. serpentine* grew in MS media supplemented with NAA [0.01 mg/L and BAP [0.5 mg/L] resulted in two buds in primary culture and four buds in subsequent sub-culture [13]. Result of a study on in vitro regenerated micro-shoots rooted found that the best was on woody plant medium[WPM] supplemented with 1.0 µM NAA [2].

Auxins and cytokinins affected the growth of shoot [cell multiplication] when planlet were cultured employing Murashige and

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Skoog [MS] medium supplemented with 10.7 μM NAA, 4.4 μM BA and 2.3 μM kinetin. [9]
Both auxin and cytokinin [CK] play complex roles in their growth function [14]. Research revealed that the role of CK in main root signal is analyzed together with the role of the shoot signal of auxine, and their interaction [15]
I was reported a high level content of alkaloid reserpine [0.096%] and ajmaline [0.092%] were recorded in callus and was followed by the roots *in vitro* plantlets.
Maximum regeneration of shoots from callus [90%] was observed in MS medium supplemented with 0.2 mg/l NAA and 1.5 mg/l BA [4]. *Rauvolfia* plant accumulates reserpine compound in the root, and concentration of total alkaloid contained in the plant *R. serpentine* reach up to 1% [16]. Plant samples claiming to contain *Rauwolfia* roots were analyzed and content of total alkaloids varied from 1.57 to12.1 mg/g dry plant material [17].
Reserpine. Chemical structure of reserpine shown in Figure 1.

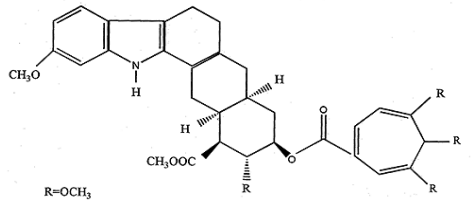


Fig 1: The chemical structure of reserpine, chemical formula: $C_{33}H_{40}N_2O_9$ [16]
Tissue culture is a culture technique to grow plant from a tiny parts of plant [explant] such as tissues, organs, or embryos.The explant is then put [planted] in a sterile artificial medium so that part of the plant is able to regenerate and differentiate into complete plants. There are several types of tissue culture includes: cell culture, embryo culture, callus culture, the culture of shoots, seed culture, meristem culture, protoplast culture. Micro propagation/tissue culture in banana plants can proliferate the number of plants in a short time, minimizing variability, and conserving of biodiversity. The success of Micro propagation depends on the plant material, macro and micro nutrients, as well as micro environment. [18]
The culture medium used in tissue culture consists of water, macro and micro nutrients, sucrose. Nutrients are essential for plant growth and development. Medium used in shoot proliferation contains about 80% in MS medium containing BAP 2.0 mg / L [19]. Another factor is the physical factors such as the status of water, evaporation, photosynthesis, respiration, growth stage, flowering and fruit formation.
Organic substances and growth regulator are very important in tissue culture acting as medium or an initiation role in cell division, cell growth and development. Growth regulator substances such as auxine and cytokinin were to be studied. Other supporting factors in tissue culture is the sterile laboratory equipment, such as autoclaves, laminar, the scale, the pH meter. Tissue culture must be maintained in a sterile condition.

2. Methodology/Materials

2.1. Material

Material used were *R.serpentina* plant explants, and growth regulators [NAA and Kinetine]. *R. serpentine* explants was taken from young leaf. The plant was available at the collection of medicinal plants of the Bogor Botanical Gardens, Bogor,West Java. Indonesia. Materials for reserpine content measurement [roots, leaf, and flower] were taken from the plant at the Bogor Botanical Garden, and the callus was taken from the in vitro culture experiment.

2.2. Implementation of study

The study was conducted within a period of three months in the Laboratory of Molecular and Cell Biology of the School of Technology and Life Sciences of Bandung Institute of Technology [ITB]. Analysis of reserpine was done with High Performance Liquid Chromatography [HPLC] with ultra violet [UV] detection conducted at the laboratory of Academy [College] of Chemical Analyst of Bogor. West Java.

2.3. Experimental methods

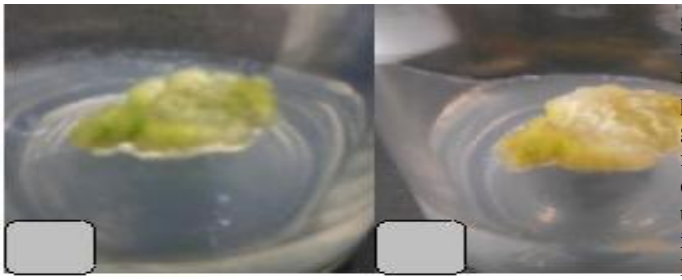
Phase I: Preparation
Preparation explants [leaves] of *R.serpentina* were washed in running water for15 minutes, and were sterilized with sodium hyphoclorate 1:41% [20% clorox] for 20 minutes. Young leaves were explanted to sterile medium of in vitro culture.
Phase II: Establishing in vitro culture**.**
The preparation of medium in vitro culture to create a medium suitable for the callus development. Murashige and Skoog [MS] medium with 25% sucrose, agar and enriched with NAA and Kinetin [as treatment arrangement of the experiment].
Combination growth regulator treatments were: 1] 1.5 ppm NAA+1.5 ppm Kinetin; 2] 1.5 ppm NAA+2.0 ppm Kinetin; 3] 1.5 ppm NAA+2.5 ppm kinetin; 4] 2.0 ppm NAA+1.5 ppm Kinetin; 5] 2.0 ppm+2.0 ppm Kinetin; 6] 2.0 ppm NAA+2.5 ppm Kinetin; 7] 2.5 ppm NAA+1.5 ppm Kinetin; 8] 2.5 ppm NAA+2.0 ppm Kinetin; 9] 2.5 ppm NAA+2.5 ppm Kinetin.
The treatments were arranged in completely randomized design with twice replications.
Statistical analysis:
The formation of callus and root were observed, and the weight of callus were measure [by weighing at an analytical balance] at the ages of one, two, three and four weeks after inoculating explants on the treated media. The analysis of variance was executed by using the DSAASTAT statistical package software.
Phase III. Reserpine Analysis.
Analysis reserpine was conducted for the sample of four weeks age of callus. Preparation of sample solution: extraction of alkaloid content was estimated by adopting the procedure utilized and developed by [20] and [4] The procedure was: take 1 g powdered of root, leaf, flower and callus [each] with 30 mL of trichloromethane in anultrasonic extraction device for 30 minutes, replication was twice and wash the residue with 30 mL trichloro methane. The extract and washing liquid were combined and filtered, then evaporated to dryness under reduced pressure in a rotary evaporator. The dried extract was dissolved in methanol, diluted to a 10mL volumetric flask. After filtering through a filter paper and a 0.45 μm membrane filter [Millipore], the extract was injected directly for HPLC analysis. In analyzing and peak determination followed the procedure adopted by [4] that the analysis were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of the peak using external standard method.

3. Results and Findings

Results of this study were found callus weight obtained from the culture in vitro [at one, two, three and four weeks of plantlet/callus age], and analysis of reserpine [of four weeks age of plantlet/callus] by the High Performance Liquid Chromatography [HPLC]. Result of analysis of callus wet weight presented in Tabel 1, and result of analysis of dry weight is presented in Table 2.

3.1. Weight of callus

Leaf explant cultured in vitro produced callus plantlet. The media was conditioned by formulating substances so as to produce callus. The medium consisted of Murashige Skooge basic medium [MS], agar, sucrose, and plant growth regulators NAA and kinetin of various concentrations. In vitro culture was maintained in sterile conditions, so that the culture facilitate the explant to grow well [Figure 2].



[a] [b] [c]

Fig 2:.Samples of *Rauwolfia serpentine* callus induction on MS medium with treatments: a [NAA 2.5 ppm and kinetin 2.5 ppm]; b [NAA 2.0 ppm and kinetin 2.0 ppm] ; and c [1.5 ppm NAA and kinetin 1.5 ppm].

Callus weight increased in line with the age as showed in the Tabel 1 and Tabel 2.

[21] said *R. serpentine* can grow optimally in medium supplemented with the growth regulators [NAA and kinetin],and the application of the growth regulators will promote development of callus on leaf explants.

Table 1: Wet Weight of Callus [gram]

MS medium+ [NAA+Kinetin] ppm	1 week [gram]	2 weeks [gram]	3 weeks [gram]	4 weeks [gram]
1,5+1,5	0.0033A	0.0289A	0.0867B	0.0944A
1,5+2,0	0.0237B	0.1003B	0.0646A	0.0369A
1,5+2,5	0.0205B	0.1098B	0.1068C	0.2406B
2,0+1,5	0.0082P	0.0605Q	0.0996R	0.1214R
2,0+2,0	0.0421R	0.0816R	0.0212P	0.0289P
2,0+2,5	0.0299Q	0.0443P	0.0475Q	0.0559Q
2,5+1,5	0.0243Y	0.0557Y	0.0378X	0.1044Y
2,5+2,0	0.0189X	0.0586Y	0.1262Y	0.0623X
2,5+2,5	0.0234Y	0.0499X	0.1379Y	0.1627Z

Note: Values followed by the same letter [at each cluster] in a column are not significantly different at P<0.05

Result of analysis of the callus wet and dry weight were shown in Table 1 and Tabel 2. The dry weight of the plant was the accumulation of photosynthesis product, it showed that the plants was in active metabolism. The highest dry weight of callus was gained from a combination of plant growth regulators 2.5 ppm NAA and 2.5 ppm kinetin, and the weight of callus was of 0.0303 grams. This shows that treatment of equal portion of NAA [auxine] and Kinetin [cytokinine] promoted callus formation.[22] noted applying cytokinine and auxine is known to promote callus formation in tissue culture.

Table 2: Dry weight of callus [gram]

MS medium+ [NAA+Kinetin] ppm	1 week [gram]	
1,5+1,5	0.00459B	0
1,5+2,0	0.00303A	0
1,5+2,5	0.00296A	0
2,0+1,5	0.00425Q	0
2,0+2,0	0.00385P	0
2,0+2,5	0.00398P	0
2,5+1,5	0.00299A	0
2,5+2,0	0.00296A	0.01626 X
2,5+2,5	0.00348B	0.

Note: Values followed by the same letter [at each cluster] in a column are not statistically different at P<0.05

Table 3: Dry weight of callus at two weeks after culture

		Kinetin [gram]	
NAA	k1	k2	k3
n1	0.0122 b B	0.0319 c C	0.0066 a A
n2	0.0040 a A	0.0035 a A	0.0106 b B
n3	0.0132 c B	0.0163 b B	0.0721 a A

Note: Values followed by the same letter in a column/a row are not statistically different at P<0.05

Table 3 and Table 4 show there are the interaction effect of Kinetin and NAA on the growth of callus. In the early stage of callus growth the weight showed slightly un-linear. In contrary, applying more kinetin and NAA showed less weight in the plants. That means the callus differentiation and growth were affected by the potential energy available in the each plantlet and initiated by the growth regulator. Culture of two weeks old was showing unconfirmed growth and differentiation as measure in the weight of callus, while further plantlet development [at 4 weeks after culture] showed different pattern of growth [Table 4]. In this stage of in vitro culture growth, the weight of callus tended to increase in line with the increase of concentration rate of the growth regulator treated and the peak was achieved at the treatment of the highest concentration [2.5 ppm NAA+2.5 ppm Kinetin].

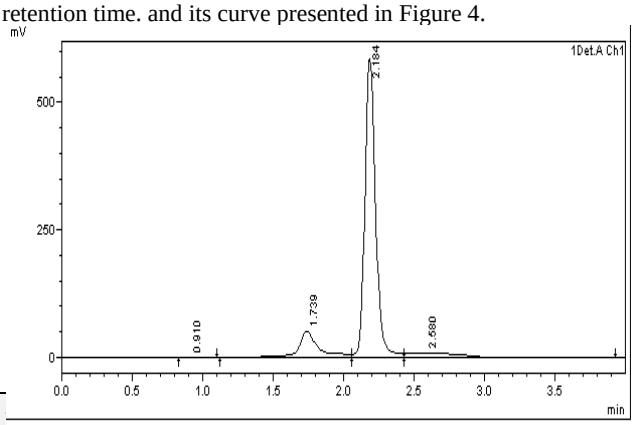
Table 4: Dry weight of callus at four weeks after culture

		Kinetin [gram]	
NAA	k1	k2	k3
n1	0.0148 a B	0.0109 a A	0.0149 b B
n2	0.0168 b Q	0.0158 b Q	0.0114 a P
n3	0.0169 b X	0.0159 b X	0.0303 c Y

Notes: Values followed by the same letter in a column or in a row are not statistically different at P<0.05

3.2 Reserpine content

Reserpine content analysis were performed by HPLC. The analysis of reserpine contents were of roots, flowers, leaves and callus. Prior to the reserpine analysis of the plant organs, the reserpine standard was determined [Figure 3].The standard content of reserpine was 0.0008 mg / L, it has shown a peak at 2.184 minutes retention time. and its curve presented in Figure 4.



Peak Table

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.910	3178	213	0.083	0.033
2	1.739	505061	51349	13.199	7.952
3	2.184	3107570	586272	81.211	90.787
4	2.580	210731	7933	5.507	1.228
Total		3826541	645768	100.000	100.000

Fig. 3: Chromatogram and peak table of standard of reserpine[0.0008 mg/L]

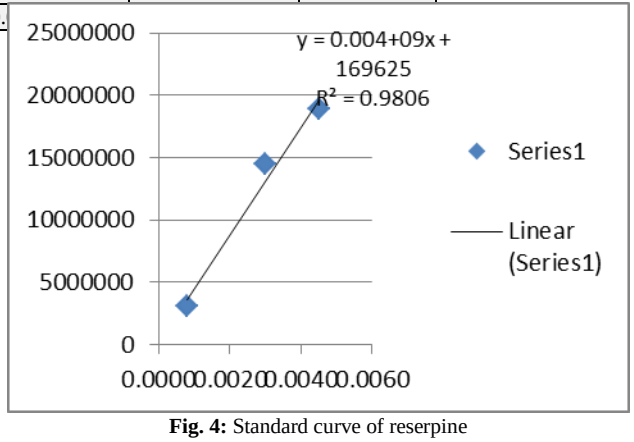


Fig. 4: Standard curve of reserpine

By using the standard analysis were calculated the contents of reserpine in the plant organs; roots, flowers, leaves and callus. Result of Reserpine analysis with HPLC is presented in Table 5, the content of reserpine in the root was 0.0021 g / L with peak retention time 2.105 minutes and its HPLC chromatogram analysis shown in Figure 5.

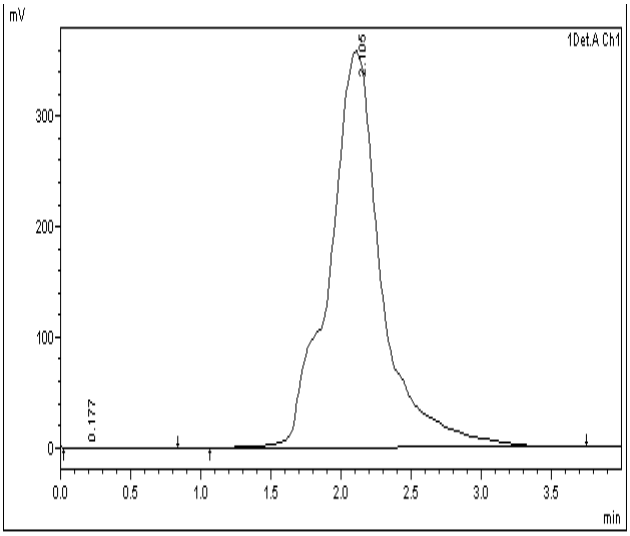
In flower organs reserpine content was 0.0015 g / L with peak retention time of 2.746 minutes, and the analysis of HPLC results are shown in Figure 6, reserpine content of the leaves is 0.0018 g / L, with peak retention time at 2.098 minutes, the results analysis of HPLC were shown in Figure 7. Callus sample of the culture at 4 weeks after culture has shown the amount of reserpine of 0.0021 g / L, and the peak retention time 2.122 minutes,the chromatogram analysis shown in Figure 8.

The contents/concentrations of reserpine in the root and in the callus were the same amount [0.0021 g / L]. The reserpine contents of roots and of callus were the highest compared to other plant organs [flowers and leaves].

The result of this study showed NAA and Kinetin application at the highest concentration [2.5 ppm NAA+2.5 ppm Kinetin] was better in promoting callus growth.And more callus will contain more reserpine compound. With this prediction of easier yielding method, in vitro culture method of reserpine production was revealed to be faster than in traditional plantation. The current effort of farmers to produce reserpine is by growing the plant with traditional cultivation. They harvest the plant and extract the reserpine compound, and they found more reserpine content can be gained in root organ. Cultivation the plant in the field may depend on environmental, meteorological and seasonal condition and need considerable workforce. While the in vitro method is conducted in laboratory or special room with a relatively less workforce and can be conducted any time.

Table 5: Result of reserpine analysis with HPLC of root, flower, leaf and callus of 4 weeks after culture [with HPLC g/L]

	Leave area	**Sample on concentration [g/mL]**
Root	9303232	0.0021
Flower	6823089	0.0015
Leaf	8076654	0.0018
Callus 4 week after culture	9138724	0.0021

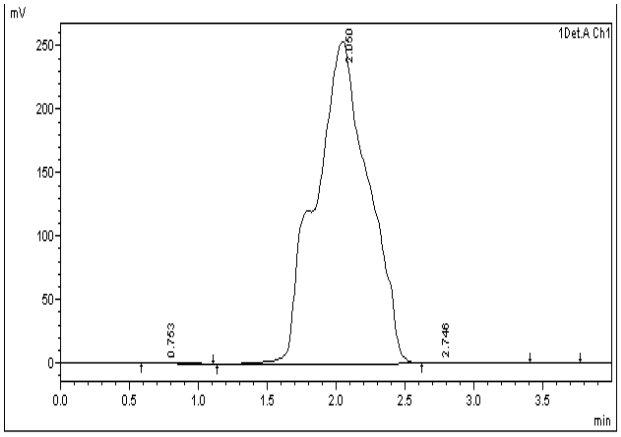


Peak Table

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.177	2439	106	0.026	0.029
2	2.105	9303232	359507	99.974	99.971
Total		9305671	359613	100.000	100.000

Fig 5: Chromatogram and peak table of root reserpine

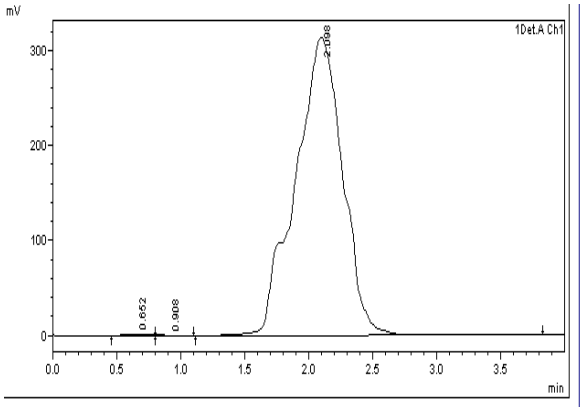


Peak Table

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.753	8395	313	0.126	0.123
2	2.050	6823089	253692	99.786	99.755
3	2.746	6069	309	0.089	0.122
Total		6837753	254315	100.000	100.000

Fig 6: Chromatogram and peak table of flower reserpine

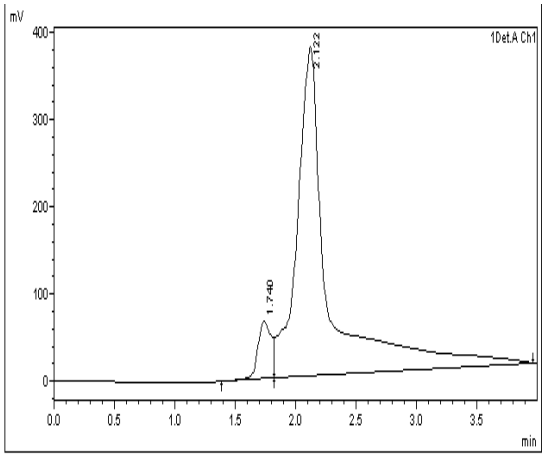


Peak Table

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.652	4471	301	0.055	0.096
2	0.908	6514	476	0.081	0.151
3	2.098	8076654	313735	99.864	99.753
Total		8087638	314512	100.000	100.000

Fig .7: Chromatogram and peak table of leaf reserpine



Peak Table

Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.740	521307	65403	6.922	14.762
2	2.122	7009560	377645	93.078	85.238
Total		7530867	443048	100.000	100.000

Fig 8: Chromatogram and peak table of callus reserpine at 4 week after culture

4. Conclusion

In vitro culture of *R. serpentine* in a basic medium Murashige and Skooge [MS], agar, sterile water, sucrose and plant growth regulators of NAA and kinetin at the concentration level [2.5 ppm + 2.5 ppm] was the best in respect of callus production. Result of content analysis of reserpine with HPLC found that the plant roots was the highest compared to other plant organs [flowers and leaves]. The content of reserpine in root and callus showed the same concentration that is 0.0021 g / L.

Production of reserpine by callus culture is faster and more effective. This fact may be the basic for recommended effort to produce reserpine compound.

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