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IN VITRO PRODUCTION OF VANILLIN FROM SUSPENSION CULTURE OF *Aerva lanata* (L.) JUSS. EX SHULTES

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ABSTRACT

In vitro propagation of *Aerva lanata* was attempted with 2, 4-D of 3 mg / ml and Kn of 2 mg /ml concentration using leaf explants. Organogenesis was recorded from leaf callus in hormone combination of BAP 5mg /ml and IAA 5 mg /ml whereas multiple shoots were induced from nodal explants on MS medium supplemented with BAP 3 mg /ml and IAA 2 mg /ml. Suspension cultures initiated from leaf derived callus of *Aerva lanata* could produce Vanillin in the presence of 2 mg/ml kinetin as an elicitor. Hairy root culture in *Aerva lanata*, established from leaf explants by cocultivating with *Agrobacterium rhizogenes* could also facilitate enhanced vanillin production in suspension culture. The vanillin produced was separated by solvent extraction and was subjected to TLC and HPLC analysis for confirming the identity.

KEYWORDS : *Aerva lanata,* Callus, multiple shoot , suspension culture, vanillin, HPLC

ABBREVIATIONS: 2,4-D - 2,4-Dichlorophenoxy acetic acid; BAP-Benzyl amino purine; IAA -Indole-3-acetic acid; Kn-Kinetin; IBA-Indole -3-butyric acid; MS-Murashige and Skoog medium; HPLC- High performance liquid chromatography

Aerva lanata (L.) Juss.ex Schultes is an important medicinal shrub of family Amaranthaceae, commonly growing in India along the wastelands. *Aerva lanata* was endowed with various chemical components such as flavonoids, alkaloids, steroids, polysaccharides, tannins, phenolic compounds, saponins, etc. (Chandra and Shastry,1990;Zapesochnaya et al., 1991) which might have contributed to its diverse uses in folklore medicine. Leaf extract of *A.lanata* was found to be very effective in the urinary risk factors of calcium oxalate urolithiasis.. Phenolic compounds form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Vanillin is one of the simple phenolic acids included in the hydroxybenzoic acid group and is mainly present in the form of glucosides. Phytochemical studies on *A. lanata* revealed that it contained phenolic compounds like vanillin and syringic acid (Zadorozhnii et al.,1986; Pervykh et al.,1993). Vanillin (4-Hydroxy-3-methoxybenzaldehyde) is one of the most important aromatic flavor compounds used in foods, beverages, perfumes and pharmaceuticals and is produced on a scale of more than 10 thousand tons per year. Vanillin is a white to pale yellow crystal with inhibitory activity against a range of microorganisms including molds, yeast, spoilage bacteria and human pathogens. Vanillin has anticancer, antioxidant, antitumor, fungicide and immunosuppressant activities.

In the present study, we have developed an efficient protocol for the rapid propagation of Aerva lanata through callus culture and nodal culture. In vitro production of vanillin as a secondary metabolite through suspension culture and hairy root culture were also attempted.

MATERIALS AND METHODS

Plant Material And Surface Sterilization

Aerva lanata was collected from local areas of Ernakulam, Kerala. The explants (leaf and node) collected from the source plant were coarsely trimmed to a size of 3 cm after removing the inflorescence and were washed under running tap water for 10 minutes followed by washing with distilled water with few drops of Tween 20. The explants were disinfected by immersing in 0.1% mercuric chloride solution for 2-3 minutes. After 4 rinses in sterile distilled water, they were inoculated onto culture medium.

Media Preparation

Basal medium (Murashige and Skoog.,1962) with 3% sucrose was used in all experiments. The pH of the media was adjusted to 5.5 - 5.8 prior to the addition of 0.8 %(w/v) difco bacterial grade agar and autoclaved at 15 lbs pressure for 15 minutes. All the cultures were maintained in the culture room at 25±2°C under 16/8 hr photoperiods.

Callus Induction

Various concentrations of 2,4-D (0.5-5.0 mg /ml) and

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2,4-D/Kn (1/. 5 mg /ml to 3 / 2.5 mg l were tested for their effect on callus induction from leaf explants. The sterilized leaf segments were inoculated on sterilized slanted media and the frequency of callus was recorded as percent of the explants forming callus

Shoot Differentiation

The light green callus obtained from leaf explants was transferred onto MS medium supplemented with BAP (5 mg /ml) and IAA (0.5-5 mg /ml at various combinations. Percentage of callus forming shoots, along with the number and length of differentiated shoots were recorded.

Multiple Shoot Induction From Node

The nodal segments of the plant were inoculated on MS medium supplemented with growth regulators viz, BAP (3 mg /ml) and IAA (0.5-2 mg /ml) of different concentrations. The number and length of differentiated shoots were recorded after 30 days of inoculation. After 45 days, the shoots were separated and inoculated on fresh medium with same hormone combinations.

Rhizogenesis

In vitro differentiated shoots measuring 2-3 cm in length were transferred to half strength MS medium supplemented with IBA (2 mg /ml) and Kn (0.5 mg /ml) for rooting.

Acclimatization

The regenerated plants with well developed shoots and roots were transferred to pots containing sterile vermiculate under diffuse light (16/8 h photoperiod) condition. Potted plants were covered with transparent polythene cover or membrane to ensure high humidity and watered every 2 days with half strength MS salt for 2 weeks. Plants were transferred to pots containing garden soil and watered with tap water.

Data Analysis

All the experiments were repeated thrice. The effect of different treatments was quantified. The data was analyzed using one-way ANOVA and means were compared using the Tukey test at the level of significance.

Suspension Culture

In order to initiate suspension culture of *Aerva lanata*, friable calluses from 2,4-D and 2,4-D/Kn with elicitor were transferred to 250 ml Erlenmeyer flask containing 50 ml of MS media. 2mg/l Kn was used as elicitor. The flasks were incubated on a rotary shaker at 120 rpm using the same conditions used for callus culture. The estimation of fresh weight and dry weight of the callus in the suspension culture was done at five days interval up to the 25th day.

Hairy Root Culture

Agrobacterium rhizogenes strain collected from MTCC, Chandigarh was used for initiating hairy root culture in leaf and callus segments in vitro (Malabadi and Nataraja., 2003). Actively growing hairy roots produced at the inoculated sites of explants were transferred to MS liquid medium supplemented with 3% sucrose. Cultures were grown on a rotary shaker at 120 rpm for 25 days and were used for analyzing the phenolic content.

Chromatographic Separation And Detection of Vanillin

The callus and the hairy roots from suspension culture was collected, dried and extracted with water. The aqueous extract was filtered and was condensed by placing in a water bath. The residue was then dissolved in n-butanol and was subjected to chromatography over a silica gel plate.

TLC was carried out on 'Merck silica gel 60' plates. The butanol extract of plant samples were spotted on the plates using capillary tubes about 2cm from the lower end of the plates (Mabinya et al.,2006) The plates were developed with Chloroform: Methanol: Formic acid (85:15:1) in a pre-saturated chromatographic chamber. The solvent was allowed to run up to 2/3rd of the plate. Developed plates were then dried in a hot air oven at 100^{o}C. Detection of the samples in the TLC plate was done under UV light of 365nm. The colour of the spots was noted and the Rf values were calculated. The spots were separately scrapped off from the plates, and the individual fraction isolated was identified by HPLC analysis at NIIST,Trivandrum. HPLC equipment used was Varian with a manual injector, a programmable wavelength photodiode array UV detector (280 nm), and C18 column packed with modified silica gel (ODS-Octadecylsilane). The linear gradient elution was carried out with solvent A (acetic acid/water (2:98 v/v)) and solvent B (acetic acid/acetonitrile/water (2:30:68 v/v)) as mobile phase.

RESULTS AND DISCUSSION

Callus production was an essential step in the

regeneration of adventitious organs (Hutteman and Priece, 1993). Callus induction in *Aerva lanata* was obtained within 2 weeks from leaf with different levels of 2,4-D and 2,4-D/Kn. Explants started callusing within 2 weeks. The callus obtained from 2,4-D alone was friable and was brown in colour. Good callus response was observed in leaf explants on MS media supplemented with 3 mg /ml 2,4-D (Fig., 1A). Green compact callus were obtained from leaf explants inoculated on MS medium supplemented with 2,4-D/Kn (Fig.,1B).Combination of 3.0 mg /ml2,4-D and 2 mg /ml Kn showed better result.(Table,1).

In the present study the organogenic efficiency of the

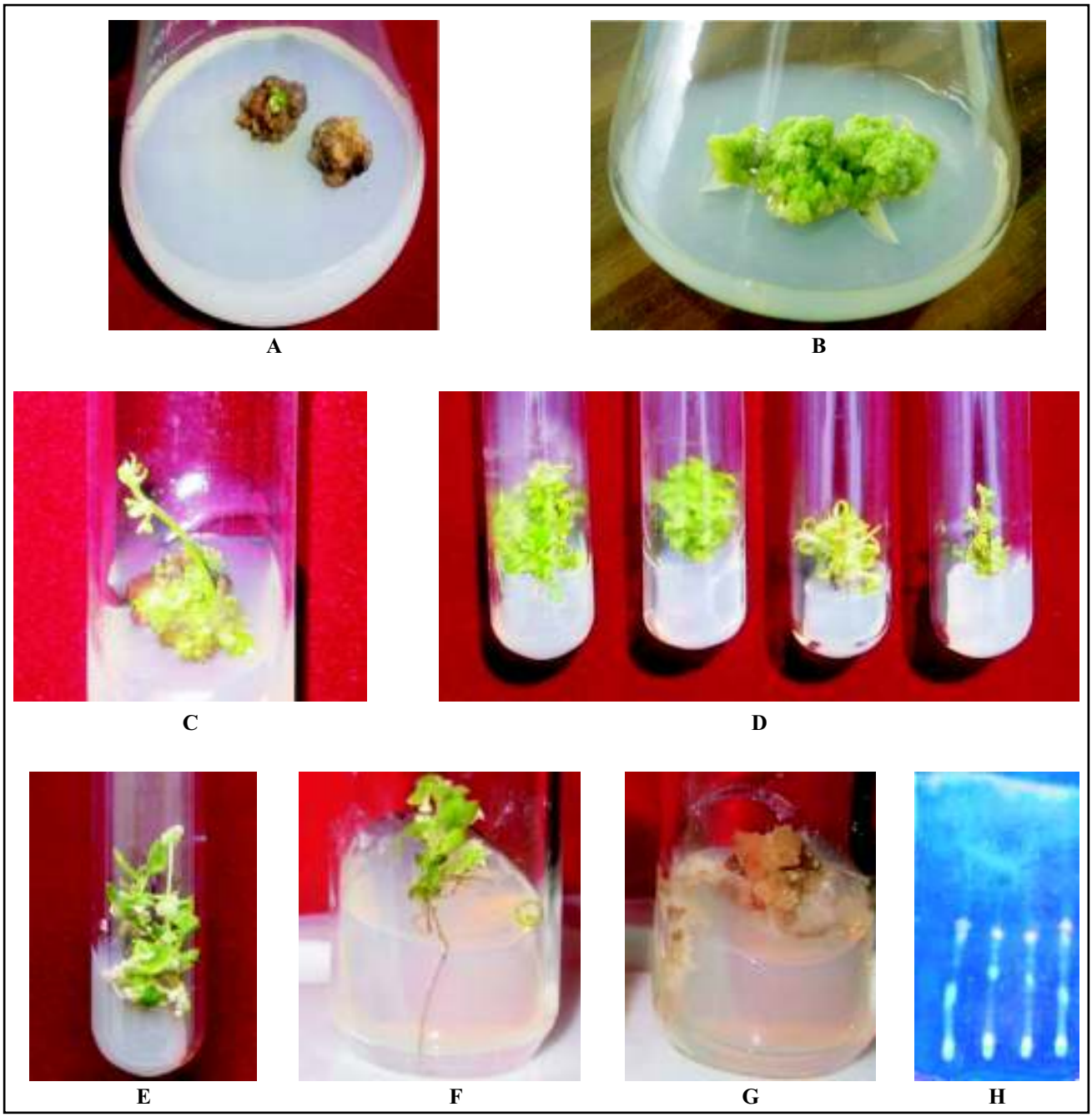


Figure 1: Callus induction and plant regeneration in *Aerva lanata*. A. Leaf calli (2,4-D) B. Leaf calli (2,4-D/Kn), C. Regenerated shoot from leaf calli, D. Multiple shoots from nodal explants, E. Shoot with inflorescence, F. Rooted plantlet, G. Hairy root on leaf callus, H. Callus extract separated by TLC and observed under UV light

Table 1: Effect of 2, 4-D and Kn on callus induction from leaf explants of *Aerva lanata* on MS medium

2,4-D (mg /ml)	Kn (mg /ml)	% of callus induction	Mean fresh weight of callus after 30 days (mg± SD)
0.5	-	40	95.73 ± 2.57
1.0	-	20	49.90±1.85
1.0	0.5	40	102.86±2.25
1.0	1.0	20	98.46±0.87
1.5	-	60	94.83±1.51
2.0	-	40	116.06±1.00
2.0	0.5	60	136.66±3.21
2.0	1.5	80	223.86±3.60
2.5	-	80	139.4±1.34
3.0	-	100	165.23±2.43
3.0	2.0	100	281.2±3.67
3.0	2.5	60	256.7±3.37

*Results are expressed as the mean of 3 replicates±SD

leaf callus was studied using combinations of BAP and IAA. The organogenic frequency was found to be low. The organogenesis was noticed after 2 months in culture. The shoots were differentiated at higher concentrations of cytokinin and auxin (5 mg /ml BAP and 5 mg /ml IAA) (Fig.,1C).

The nodal segments cultured on MS media supplemented with combinations of BAP and IAA showed axillary shoot proliferation (Fig.,1D). The frequency of response was more with BAP 3 mg /ml and IAA 2 mg /ml (Table,2). Combination of BAP and IAA substantially increased the frequency of responding in nodal explants (Fig.,1E).The results showed that the cytokinin (BAP) was very effective for shoot regeneration. The same results have been proved in some other species of Amaranthus. (Lupi et al.,1987) Nodes were better and more convenient explants for micropropagation and were ideal for maintaining genetic stability. The root formation was resulted from high concentration of auxin and lower concentration of cytokinin. High frequency of rooting was observed at 2 mg /ml IBA and 0.5 mg /ml Kn (Fig.,1F).

Table 2: Effect of BAP/IAA on multiple shoot formation from nodal explants after 30 days on full MS medium

BAP (mg /ml	IAA (mg /ml	% of shoot induction	Mean no. of shoots after 30 days	Mean no. of shoot length after 30 days
3.0	0.5	80	3.33 0.57	1.73 0.25*
3.0	1.0	40	2.00 1.00	0.26 0.05
3.0	2.0	100	4.66 0.57	0.46 0.05
3.0	3.0	20	1.00 1.00	0.23 0.05

*Results are expressed as the mean of 3 replicates±SD.

The leaf callus inoculated into the suspension culture showed cell separation and multiplication of the callus. The growth of the cells in suspension culture was determined by estimating the fresh weight and dry weight of callus (Fig., 2& 3). A steady increase in both the estimations showed gradual growth of the callus in the suspension culture. The callus multiplication was more in the medium containing the elicitor than the control.

Kinetin has been already reported as an elicitor to induce the production of vanillic acid in the suspension cultures of Vanilla panifolia. Kinetin induced catechol-4-methyltransferase activity, catalyzing the formation of 4-methoxycinnamic acids, which were shown to be the intermediates of hydroxybenzoic acid biosynthesis in Vanilla planifolia (Funk and Brodelius., 1992)

The presence of vanillin as a secondary metabolite present in the callus suspension culture of Aerva lanata was first detected by preparative TLC. The thickness of the plate, polarity and choice of solvent are important factors in the separation of phenolic acids.The spots were found to be more intense and bigger in 20th day callus extracts compared to other days.Vanillin was identified based on their colour and Rf values(Fig.,1H).

The hairy root formation was resulted within 2 weeks under dark condition. White colored roots were formed at the Agrobacterium injected parts of the explants (Fig.,1G). After 2 months they were transferred to suspension culture medium. Root extracts were collected and the presence of vanillin was confirmed by TLC and HPLC. The HPLC analysis result of the extracts from suspension culture of both callus and hairy root were

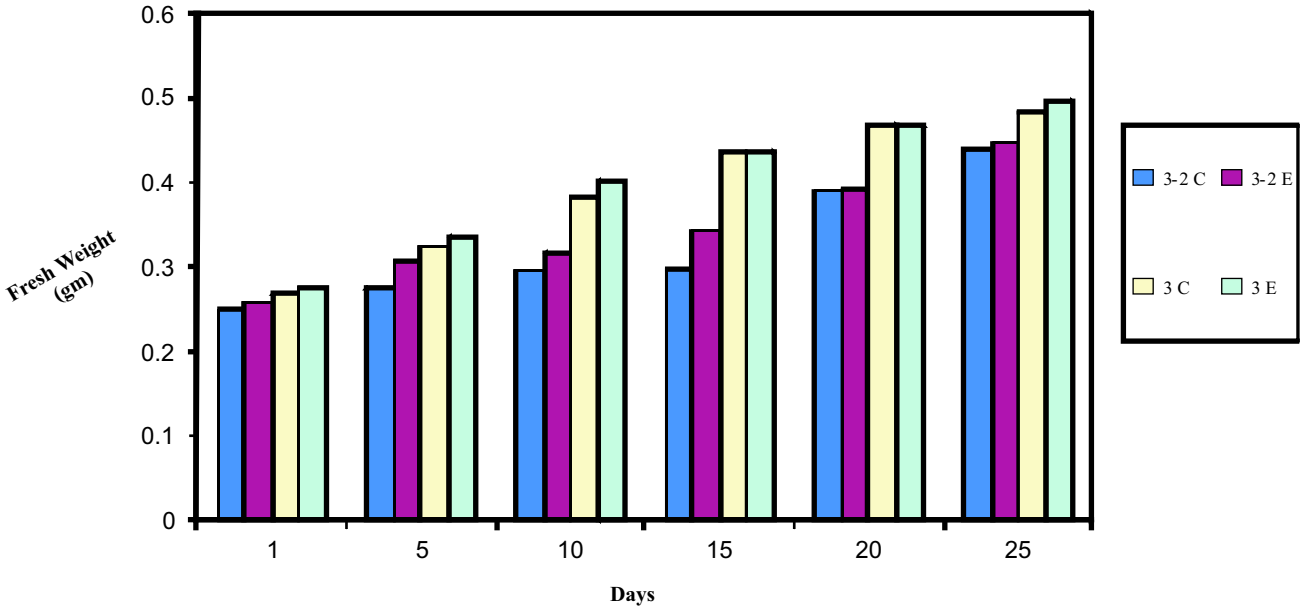
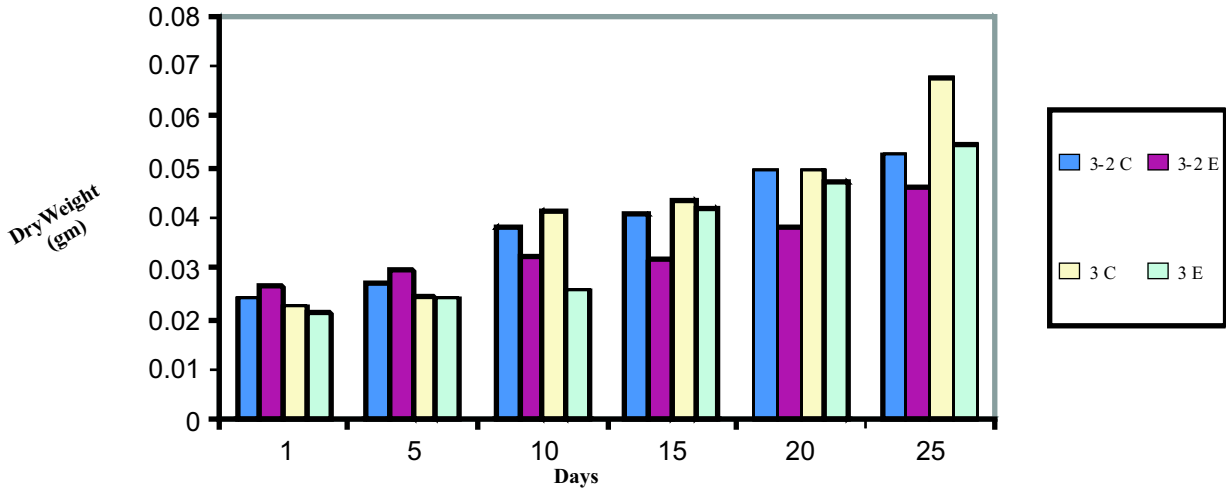


Figure 2: Fresh weight of callus from the suspension culture of *Aerva lanata* inoculated with leaf derived callus



3-2 C : 2,4-D 3 mg /ml + Kn 2 mg /ml
3-2 E : 2,4-D 3 mg /ml + Kn 2 mg /ml + Elicitor
3- C : 2,4-D 3 mg /ml
3-E : 2,4-D 3 mg /ml + Elicitor

Figure 3: Dry weight of callus from the suspension culture of *Aerva lanata* inoculated with leaf derived callus

compared with vanillin standard. (Fig., 4, 5 & 6).

The results obtained in the present work were promising and suggested a viable methodology for the micropropagation of *Aerva lanata* and also for the production of vanillin as a secondary metabolite. Once appropriate technology is developed large-scale production of vanillin from these cultures can be. The present findings could also be utilized commercially for the in vitro propagation and multiplication of *Aerva lanata.*

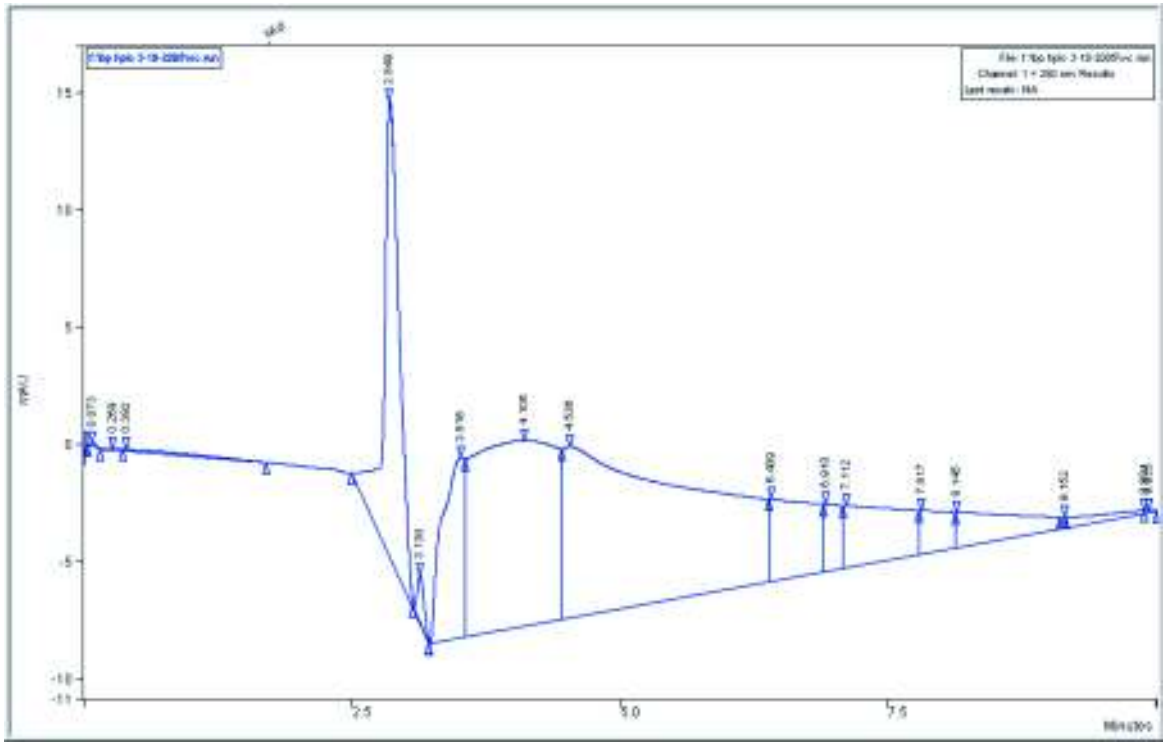


Figure 4: HPLC of vanillin standard

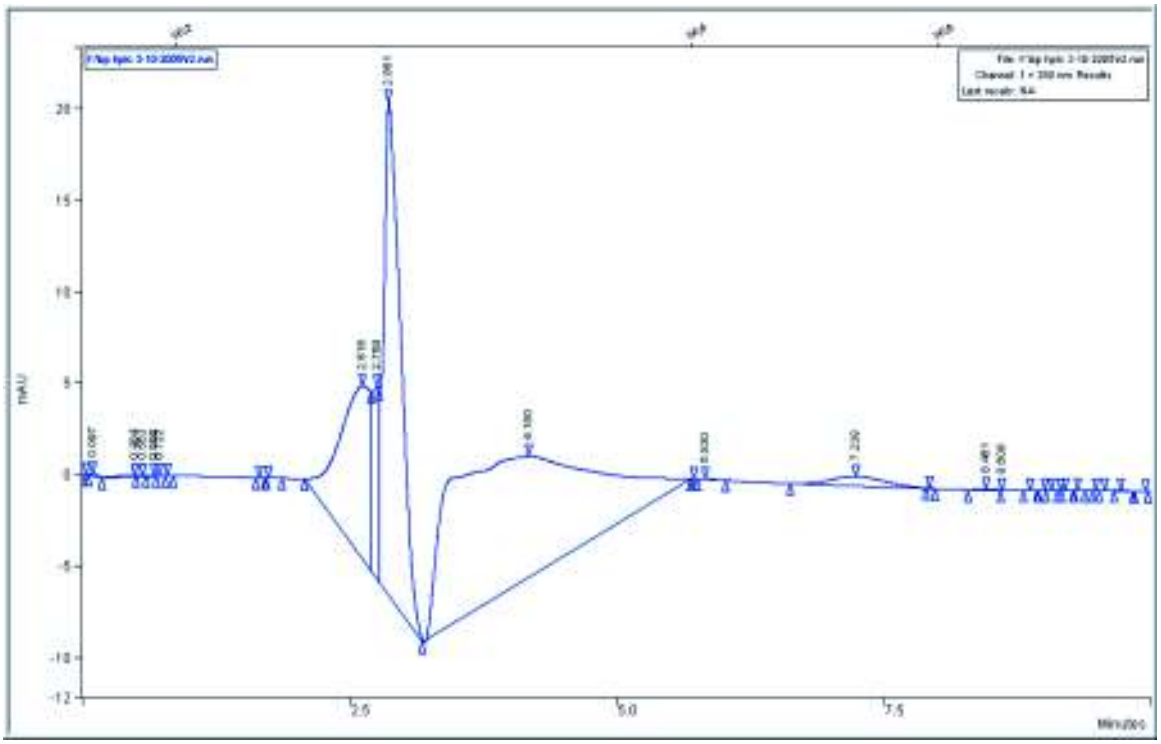


Figure 5: HPLC of the extract of the suspension culture induced by the leaf callus of *Aerva lanata*

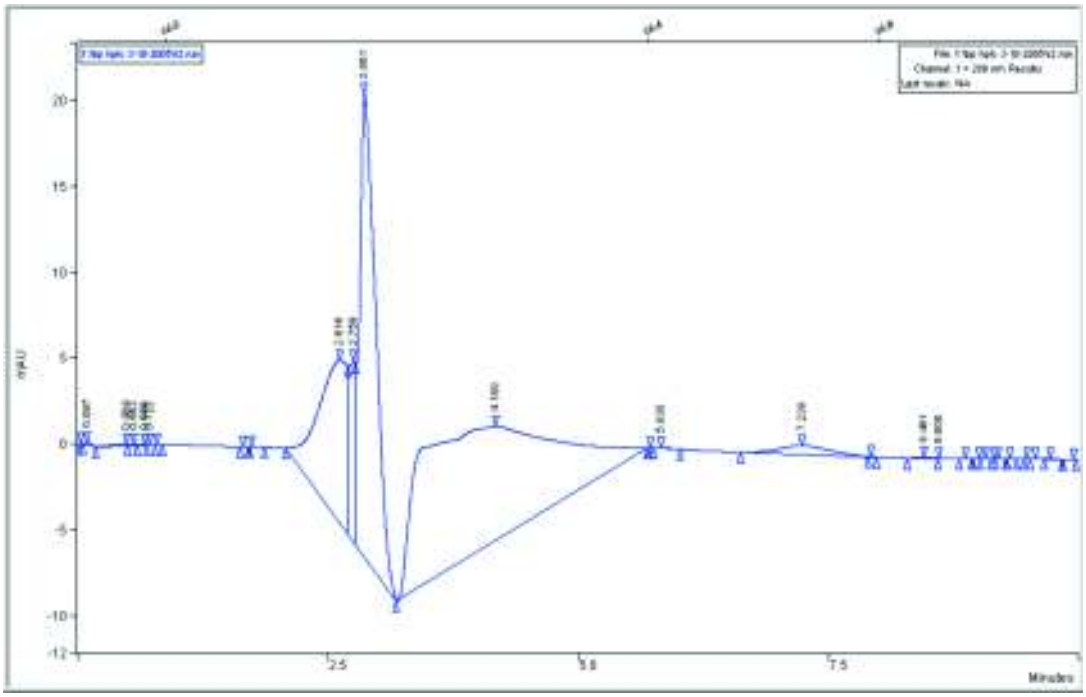


Figure 6: HPLC of the extract of the suspension culture induced by hairy root culture of *Aerva lanata*

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