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Enhanced Production of Aloe Mannan using Plant Biotechnology

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Abstract

Plant tissue culture is a promising technology, especially for the multiplication and production of novel and improved plants species and for an increased biosynthesis of products of industrial and medicinal value from vegetative resource. The development of a callus route was availed to investigate the in vitro plants for enhanced production of the bioactive polysaccharide acemannan in Aloe barbadensis plants. 8 mg/L NAA and 1.5 mg/L BAP with four different carbon sources (sucrose, mannose, glucose and galactose) and five concentrations (2, 3, 4, 5 and 6%) and kept in dark conditions for 1 month. The production media and light conditions played a vital role to increase the acemannan levels in stress conditions. The investigation revealed quite interesting results as the acemannan yield increases several fold in the in vitro regenerated callus cultures and Aloe barbadensis plant extracts analyzed for acemannan content by HPLC. Cultures incubated in media having 3 % mannose sugar produced the highest concentration of acemannan i.e 0.95 mg/mL, which revealed a remarkable enhancement of acemannan production as compared to the in vivo production in Aloe vera plant in soil conditions giving yield of 0.43 mg/mL acemannan. Cultures in media having 3% sucrose only increased 2% production of this compound in vitro giving 0.44 mg/mL yield. On the contrary, when glucose and galactose were added in the culture media the yield of acemannan was suppressed in all the tested concentrations.

Keywords: Aloe Barbadensis, Acemannan, Carbohydrates, HPLC

Introduction:

Aloe vera leaves are made up of a thick epidermal layer which surrounds the mesophyll tissues, which are differentiated into thin-walled parenchyma cells and collenchyma cells. The inner parenchyma is composed of parenchymatous tissues which comprises of 0.5% solid matter and 99% water. In 1999, Femina and co-workers observed that polysaccharides constitute the major portion of the Aloe vera parenchyma having two main kinds of polymers: a storage polysaccharide "acemannan" was located in the protoplasts of cells and a broad range of polysaccharides formed matrix of the cell wall. Aloe vera gel is obtained from the leaf by the process of filleting then subjected to homogenization, alcohol extraction to yield a complex high molecular weight carbohydrate. In the early 1980's, an acetylated polymannose, acemannan, was identified as an active component of Aloe vera gel (Johnson et al., 1989). This carbohydrate was commercially designated as acemannan by the United States (US. Patent WO/1993/008810). According to the literature reports, significant seasonal and cultivar variations affect the quantities of mannose residues in the storage polysaccharide within the parenchymal cells, leading to huge fluctuations in the polysaccharide composition of A. vera fillet (Femenia et al., 1999). A wide range of in vivo and in vitro immunostimulatory activities of polysaccharides isolated from Aloe vera plant gel have been documented (Kardosava, 2006; Akaberi et al., 2016). Acemannan is considered to be the key biologically active substance found in the Aloe vera plant gel (Lee et al., 2001; Hamman, 2008; Mahor & Ali, 2016). Acemannan, a β-(1, 4)-linked polydispersed, highly acetylated mannan has an average molecular weight of 1000 kDa (Turner et al., 2004; Chow et al., 2005). The quality of Aloe vera gel preparations can be accessed by 1H NMR technique, which has proven to be an essential tool for observing the presence of the bioactive polysaccharide acemannan. According to Manna & McAnalley (1993), acemannan is a linear polysaccharide consist

of β-(1→4)-linked mannan partly acetylated in positions 2, 3 or 6, having galactose units attached to C6 forming some side chains. In H-NMR spectrum, the multiple peaks of acetyl groups produce a characteristic signal from 2.0 to 2.3 ppm, which can be reflected as the benchmark of Aloe vera (Bozzi et al., 2007).

Acemannan Isolation and Purification Studies

Modern science in the early 20th century was applied in order to recognize and identify the active ingredients in the design cultivation of Aloe vera, processing and harvesting techniques to store, preserve and familiarize Aloe vera into larger commercial markets. The Aloe active ingredient acemannan having immense therapeutic potential and applications has been subjected to various, isolation, separation and purification studies (Acemannan Review by Sierra-Garcia et al., 2014). Assays of acemannan were developed and purified through various chromatographic techniques including HPLC (Okamura et al., 1996, Talmadge et al., 2004; Chandran et al., 2017), gel permeation chromatography (McAnalley, 1993; Femenia et al., 1999; Choche, et al., 2014) and size exclusion chromatography (Turner et al., 2004; Alonso et al., 2012; Salah et al., 2017).

Earlier studies have shown that acemannan is not steady under various conditions utilized in the manufacturing process including acid, heat, enzymes discharged from cells while processing (Femenia and Garcia, 2003; Minjares-Fuentes et al., 2017). Unfortunately due to improper manufacturing processes used by many Aloe product manufacturers, acemannan is partially and sometimes fully removed, having Aloe products with little or no acemannan (Turner et al., 2004). Moreover, currently most manufactures do not assay for acemannan content in their final products. Instead the presence of adulterants, impurities, and preservatives have been found in many leading Aloe based commercial products (Bozzi et al., 2007). The varying and inconsistent quality of Aloe vera preparations have resulted in numerous conflicting scientific studies and introduction of several products into the market place of variable quality causing conflicting consumer experiences, and collectively emerging in controversy about the true medicinal benefits of Aloe vera.

Materials And Methods

Selection of Mother Plant

The Aloe vera species were collected from various cities of Sindh, Punjab, Baluchistan and Khyber Pakhtunkhwa and planted in the field of H.E.J. Research Institute of Chemistry, University of Karachi. Screening of the plants was done to identify disease free and healthy specimens. Best mother plants were selected on the basis of high yield of leaf biomass and were evaluated physically for the absence of any contamination. Plants are decontaminated by spraying them with agents such as pesticides, fungicides or insecticides. Preferred fungicides for the pretreatment of the mother plant include Benlate and Topsin M at a concentration of about 0.05% to 0.2%.

The plants were tested for high acemannan content using standard analytical techniques including TLC, HPLC, FT-IR spectroscopy and ^{1}H-NMR spectroscopy. The identification of known constituents was done by comparing with their acquired and reported spectrums.

Acemannan Isolation and Purification:

Plant collection for acemannan analysis

Four different varieties of Aloe vera were collected from Karachi, Islamabad, Quetta and Hyderabad. Botanical identification was done by the Botany Department, University of Karachi. Their names with their designated codes are: Aloe alfredii (A1), Aloe barbadensis (A2), Aloe dwetii (A3), Aloe bellatula (A4). From 2 kg leaves of each variety 30 mg of gel extract was subjected to various extraction, isolation and purification steps to obtain acetyl mannan dry powder.

Isolation and Purification

Aloe vera gel extracts of 2 kg leaves of different Aloe varieties were provided by collaboration of P.C.S.I.R. laboratories, Karachi, Pakistan. The gel samples were kept at 4º C for 2 hrs. Acetylated mannans in the gel of polysaccharides were extracted and isolated by alcohol precipitation method (McAnalley, 1993), with some modification. The clear inner gel was homogenized and most of the pulp removed. The viscous gel was brought to an acidic pH of approximately 3.20 with dilute HCl, which solubilizes the oxalates and lactates of calcium and magnesium that are normally present. Then extraction of the gel was done for 5-6 hours with 4 volumes of 95% ethanol at room temperature and kept overnight at 4^{0} C for maximum precipitation of the bioactive polysaccharide. The fibrous floating particles were removed and the water/alcohol mixture was discarded. Then centrifugation (Hareus, Biofuge) was done at 10,000×g for 30 min at 4^{0} C to obtain the solid precipitate. Most substances such as oligosaccharides, organic acids, monosugars, inorganic salts and anthraquinones which are soluble in the water/alcohol mixture were eliminated in the process. The deposit was then dissolved in deionized water and passed through 0.22 µm filters (Millipore), using high pressure vaccum pump filteration (Millipore), lyophilized (Freeze Dryer Trio Science, Model TR-FD- BT-50) and stored at -20^{0} C until further analysis.

Characterization and Qualitative Analysis

Acetylated polymannose, was characterized using TLC, HPLC, FT-IR spectroscopy and ^{1}H- NMR analysis.

Fourier Transform Infrared (FT-IR) Spectroscopic Analysis

The functional groups of acemannan absorb at characteristic infrared frequencies. Infrared spectroscopy therefore is an important method to characterize this material.

Chemicals

Infrared grade potassium bromide (KBr) powder (Merk).

Instrumentation

Broker Fourier Transform infrared (Ft-Ir-8900) spectrophotometer model Vector-22 equipped with a Pentium 2 Computer and a HP 640 C Deskjet printer/plotter.

Sample Preparation

Acemannan was pre-ground to fine powder by using Wiley Mill (Thomas Scientific CO.) and a screen which allows particles smaller than 60 mesh size. 1mg pre-ground sample was mixed with 30 mg of dry KBr powder. The mixture was reground by hand using agate mortar and pestle to a homogeneous material. Then the sample was pressed into a transparent disc using a hydraulic jack (Riken Power) at a pressure of 40,000 psi. The disc was scanned from 4000 cm ^{1} to 400 cm. a multiple scan (20 scans) was performed

with a 2 cm resolution to improve signal to noise ratio.

Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectra were recorded in D2O on Bruker AV 500 NMR spectrometer using the Windows top spin 2.1 operating system, at 500 MHz, for ^{1}H-NMR.

Acemannan determination from Aloe vera calli under carbohydrate stress conditions.

Aloe plant calli were subjected to different carbohydrate stress conditions in order to study the effects of various concentrations of carbohydrates on acemannan production and enhancement.

Calli were sub-cultured on MS media supplemented with 8 mg/L NAA and1.5 mg/L BAP, having Sucrose (2-6%), Mannose (2-6%), Glucose (2-6%) and Galactose (2-6%). Each treatment had 5 replicates. After 30 days of incubation in dark conditions calli were pooled, harvested and fresh weight was calculated. The callus crude extract was obtained by the method described by McAnalley, 1990 with some modifications. Briefly, each callus sample was ground to powder by liquid nitrogen. The samples were dissolved in water and homogenized to remove most of the pulp. The pH of the callus sample extracts was adjusted to 3.20 with dilute HCl. Then extraction was done for 4-5 hours with four volumes of 95% ethanol at room temperature and kept overnight at 4 ºC for maximum precipitation of the bioactive polysaccharide. This was followed by centrifugation (Hareus, Biofuge) at 10,000 × g for 30 min at 4 ºC. The supernatant was siphoned off and deposit was dissolved in deionized water and passed through 0.22 µm filters (Millipore), using high pressure vaccum pump filteration (Millipore), lyophilized (Freeze Dryer Trio Science, Model TR-FD-BT-50) and stored at -20 ºC until further analysis.

TLC Analysis

The methanolic extracts were studied and analyzed by reverse phase thin layer chromatography (RP-TLC) using RP-18 silica gel coated glass plates by co-chromatography with acemannan standard. The plates were developed in a solvent system of methanol: water (2:8), dried and detected using ceric sulphate spray reagent.

Recycling Preparative High Performance Liquid Chromatography:

Reagents and Chemicals

Methanol and acetonitrile (HPLC grades) were purchased from Fisher Scientific. AMP Floracel ®, USA, containing 90 mg powder of Aloe mucilaginous polysaccharides (AMP) extracted from Aloe vera plant was used to obtain Aloe vera mannan used as reference standard. Commercially available Aloe vera samples do not have acemannan in purified form. Thus an isolation and purification method of this bioactive compound was developed and optimized by using Recycling Preparative High Performance Liquid Chromatography HPLC Waters Alliance (ODS-M-80). The mobile phase was MeOH:H2O (20:80) with flow rate 3 mL/min, to obtain acemannan (8; 10 mg; retention time 40 min). Estimation of acemannan was done by comparing spectras of FT-IR and ^{1}H-NMR with the literature reports.

Chromatographic Conditions

The dried crude extracts were dissolved in solvent MeOH:H2O with the ratio of (20:80) and extraction of samples was done by sonication (Sonicator from Fisher Scientific) for 10 min. Samples were vortexed by using Vortex Mixer (Fisher Scientific). Then the extract was filtered with a 0.45 µm syringe filter, prior to HPLC analysis. The samples were further purified by preparative recycling HPLC (Waters Alliance, (ODS-M-80) and detector set at wavelength of 210 nm. The mobile phase was MeOH:H2O (20:80) with flow rate of 3 mL/min to obtain acemannan (8; 10 mg; retention time 40 min).

Quantitative Analysis

Instrumentation

The HPLC analysis of standard acemannan and sample extracts was carried out on a Waters Alliance 2695 HPLC system with automated gradient controller, 2996 photodiode array detector connected to a computerized data station using Waters Empower Software Build 1154.

Sample Preparation

Sample preparation is a very important step in the development of standard analytical procedures required for the analysis and standardization of herbal and botanical preparations, which must be effective and reproducible. The protocol for the extraction of Aloe vera samples was optimized and different parameters such as solvent systems, HPLC columns, time and temperature were carefully studied in order to validate the efficiency of the extraction method.

For quantitative analysis, dried plant extracts were dissolved in MeOH:H2O (20:80) and extraction of samples was done by sonication (Sonicator from Fisher Scientific). Samples were vortexed by using Vortex Mixer (Fisher Scientific). Then the extract was filtered with a 0.45 µm syringe filter, prior to HPLC analysis. The mobile phase (80% water and 20% methanol) was sonicated for 20 min for removing air bubbles prior introduction to HPLC system.

Chromatographic Conditions

Chromatographic conditions were optimized to obtain the best separation of standard peak, in the standard mixture as well in the Aloe vera extracts. Stationary phase used was the reverse phase C-18 (250 × 4.6 mm) and the column temperature was 37 ºC. The mobile phase consisted of 80% water and 20% methanol. Analysis was carried out by isocratic elution at a flow rate of 1.0 mL/min, with injection volume of 20 µL. The total analysis run time of each sample was 10 min, which was followed by a 5 min wash with 100% methanol. The peak of acemannan standard and all the samples were detected at a wavelength of 210 nm. For correct identification of the peaks the comparison of the UV spectra and retention times was done with standard compound.

Preparation of Standard Calibration Curve

Standard stock solution was made by diffusing 10 mg of purified acemannan standard in 10 mL of 20% methanol and 80% water to make solution. Then the solution was vortexed and filtered by using 1 mL syringe and 0.45 micron filter assembly. Then different concentrations in the range of 0.1-10 mg/10 mL were prepared by serial dilution for HPLC-UV analysis. The peak areas of each concentration were recorded and the standard calibration was constructed by plotting the concentration on X-axis and corresponding peak area was plotted on the Y-axis.

Results And Discussion

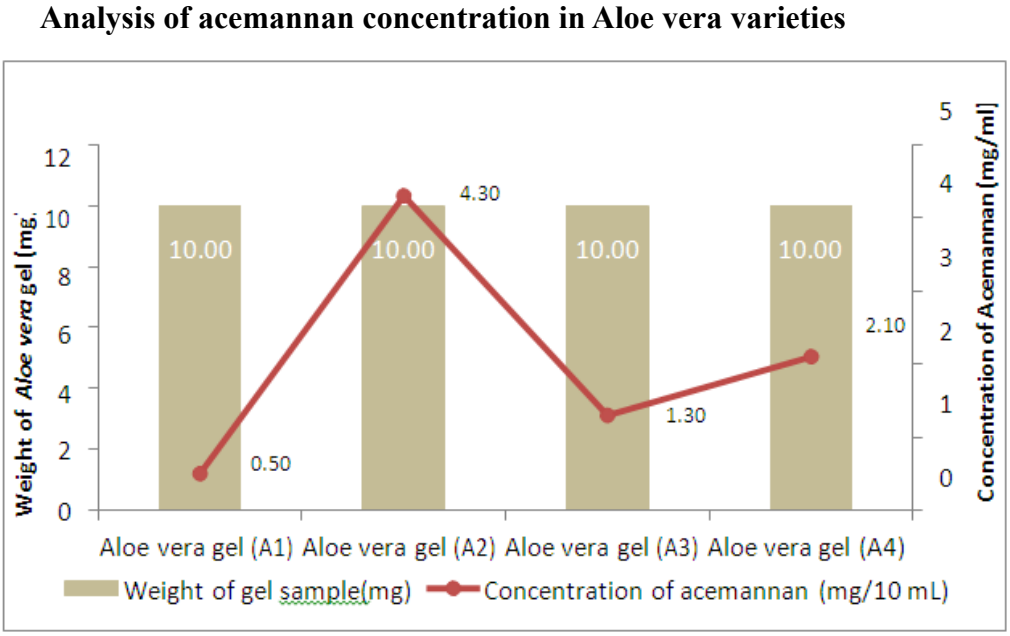
In the present study an elite variety of Aloe barbadensis, having

high acemannan content was investigated and exploited for micropropagation and enhanced production of its bioactive compound acemannan. Attempts were made to explore the potential of a highly efficient multiplication method based on callus induction and regeneration of A. barbadensis. The effect of various factors such as light/dark conditions, various types and concentrations of plant growth regulators and carbohydrate sources, on the efficiency of the multiplication system as well as on acemannan production and enhancement was evaluated.

Selection of Elite Mother Plant

Four different varieties of Aloe vera were collected from different cities of Pakistan. From 2 Kg leaves of each variety 30 mg of gel extract was subject to various isolation and purification steps to obtain acetyl mannan dry powder. The concentration of acemannan in four different varieties was studied which demonstrate the varied specie production of this anti-cancer molecule having numerous pivotal biological activities. All varieties were taken from field as described in experimental section. From all the varieties tested, Aloe barbadensis (A2) had maximum quantity of acemannan i.e. 0.43 mg from 10 mg of the gel extract analyzed, whereas minimum concentration was in A. alfredii, which was 0.05 mg as represented in Graph 1.

Graph 1: Shows the analysis of acemannan concentration in different Aloe varieties



FT-IR Spectrum Analysis

The IR spectra summarized clearly indicates the presence of the bioactive polysaccharide acemannan in the Aloe varieties (Table 1 and Figure 2) and in Aloe barbadensis callus cultures and in vitro regenerated plants from callus (Table 2), which was referred against standard A, (Acemannan Immunostimulant (AMP Floracel ®, USA), **Figure 1**.

Figure 1: FT-IR spectrum of Aloe vera standard

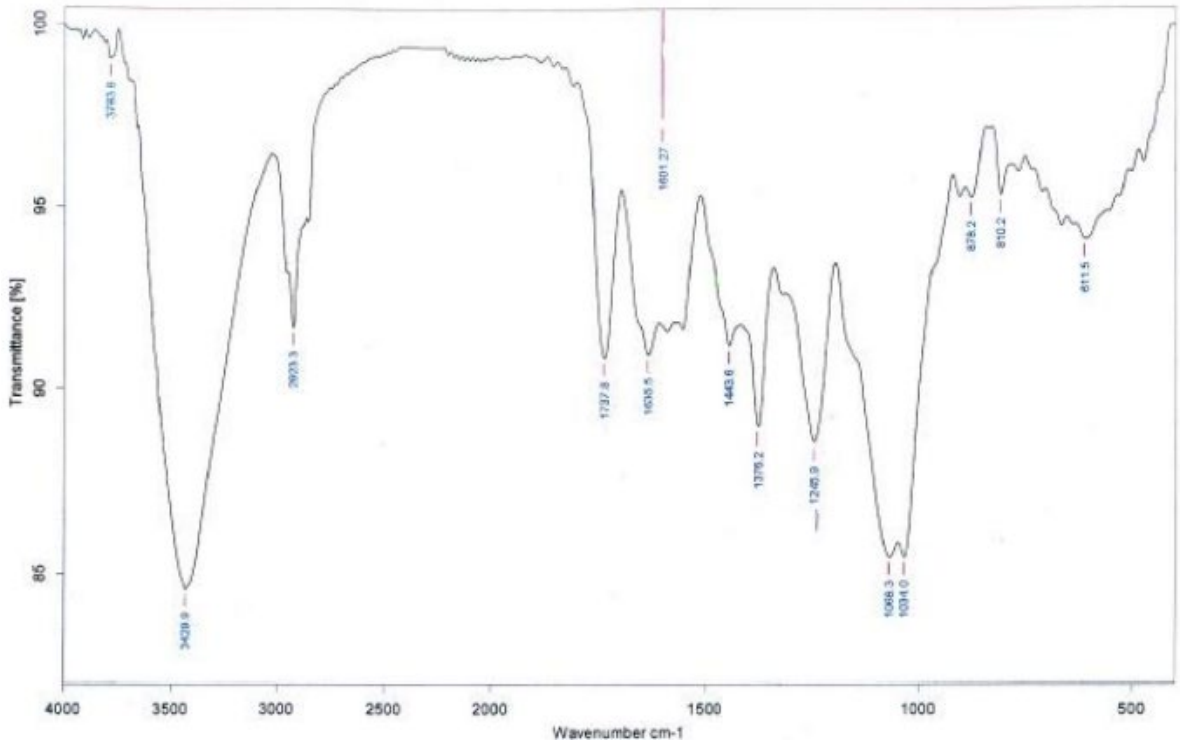


Figure 2: FT-IR spectrum of Aloe vera plant gel

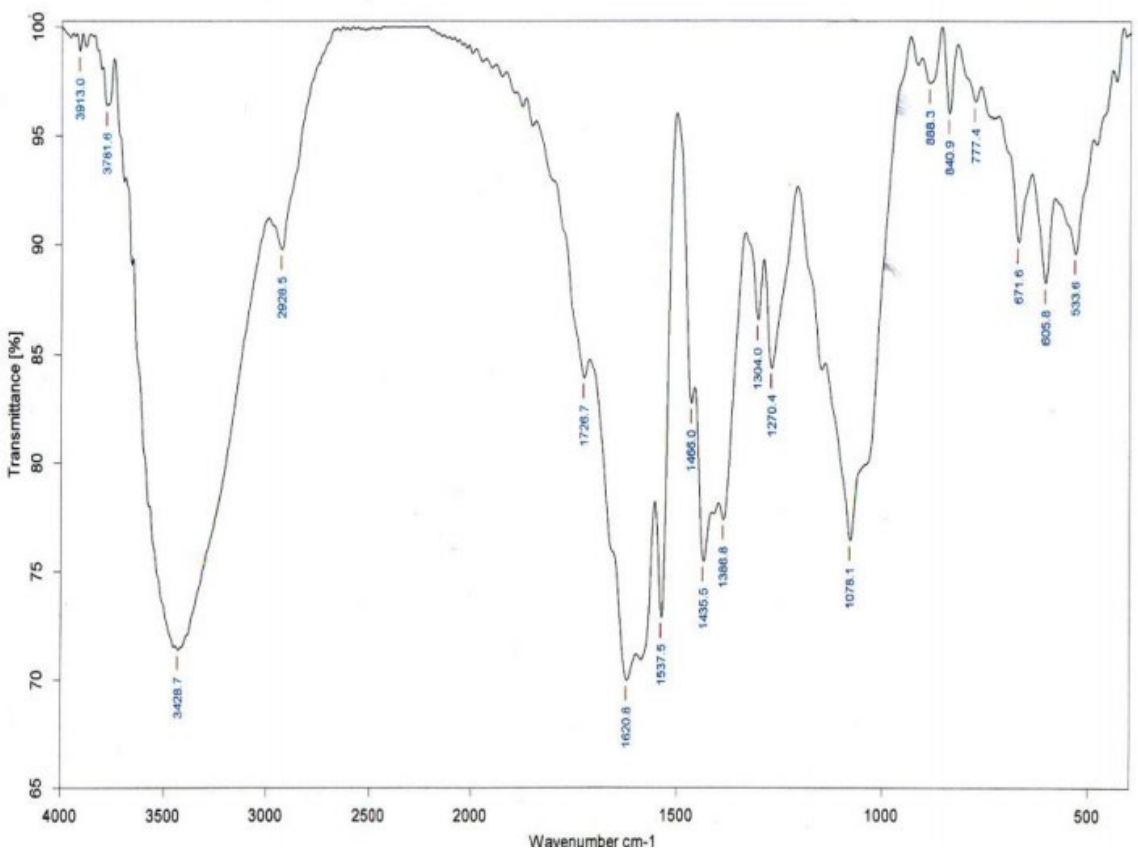


Table 1: Shows the wavenumber (cm^{1}) of acemannan in Aloe species

	Wavenumber (cm^{-1})				
	A	**A 1**	**A 2**	**A 3**	**A 4**
C-O (Pyranose ring)	1068.3	1077.7	1078.1	1091.6	1090.2
O-H	3429.9	3415.7	3428.7	3364.3	3409.6
C-O-C	1245.9	1272.1	1270.4	1242.3	1240.0
C (Carbonyl)	1737.8	1724.7	1726.7	1720.0	1700.0
C-H (Bending)	1376.2	1304.6	1386.8	1323.9	1320.0
C (Acetyl)	1635.5	1663.4	1620.8	1645.5	1662.2
C-H (Stretch)	2923.3	2924.2	2928.5	2928.6	2925.2

Table 2: Shows the wavenumber (cm^{1}) of acemannan in callus cultures and in vitro regenerated Aloe barbadensis plants from callus

	Wavenumber (cm^{1})				
	A	**C7A (D)**	**C7A(L)**	**C7A* (D)**	**C7A* (L)**
C-O (Pyranose ring)	1068.3	1076.2	1078.1	1039.6	1076.2
O-H	3429.9	3452.3	3446.6	3413.8	3456.2
C-O-C	1245.9	1272.9	1272.9	1250.0	1276.8
C (Carbonyl)	1737.8	1700.0	1700.0	1700.0	1700.0
C-H (Bending)	1376.2	1394.4	1305.7	1300.0	1396.4
C (Acetyl)	1635.5	1600.0	1600.0	1645.2	1600.0
C-H (Stretch)	2923.3	2923.9	2923.9	2923.9	2925.8

The spectrum of bioactive polysaccharide acemannan (A) demonstrates a strong absorption due to O--H stretching about 3429 cm^{-1}. The carbonyl and C--O--C stretches of acetyl group are located near 1737 and 1245 cm^{-1} respectively. The strong single band of C--O--C system is indicative of O-acetyl group bonded equatorially to the monomer unit. Thus, FT-IR spectra mentioned above clearly indicates the presence of this bioactive compound (Prerak Patel et al.,2012; Ray, et al., 2013).

Determination of Acemannan in conventionally propagated, in vitro regenerated Aloe vera plants and calli

In the present study the genetic variability of regenerated plants and conventionally grown Aloe barbadensis was investigated and exploited for the enhanced production of bioactive acemannan. The maximum concentration of acemannan as summarized in

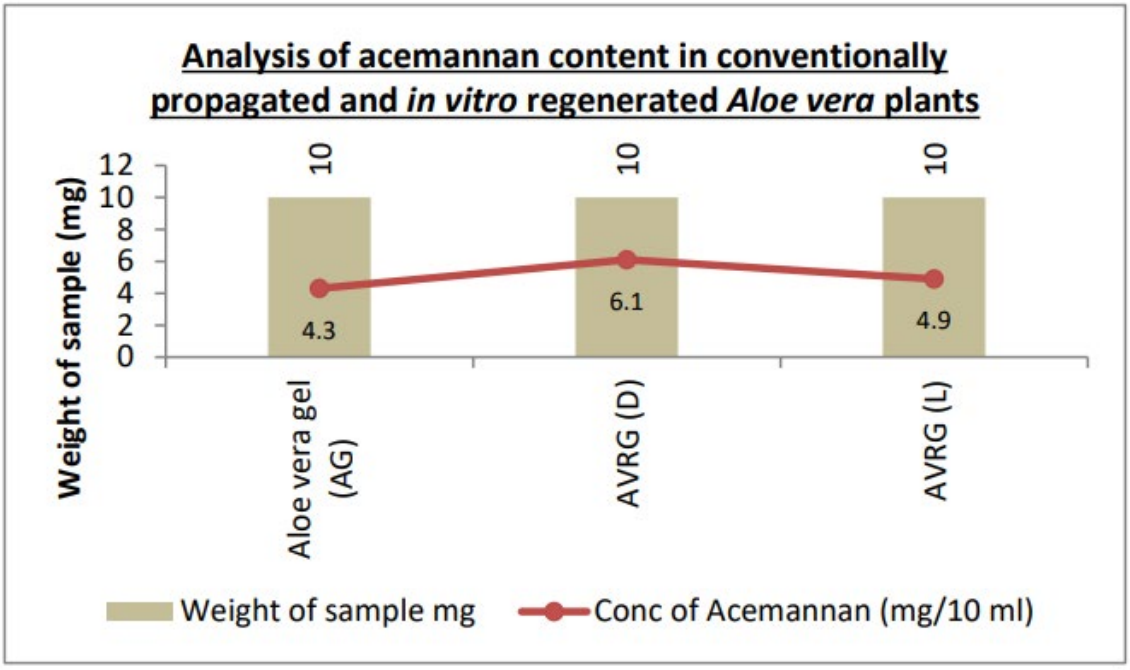
Table 3 is observed in the in vitro regenerated Aloe barbadensis plants cultured in media having 3% sucrose under dark conditions i.e 6.1 mg/10 mL as compared to the soil grown and tissue cultured plants and callus cultures under light conditions.

Table 3: Shows analysis of Acemannan concentration in coventionally propagated Aloe barbadensis plant (AV 4), in vitro callus cultures (C7A) and regenerated plant from calli (C7A*), under both light and dark conditions

Media Codes	Weight of sample (mg)	Conc. of acemannan (mg/10 mL)
AV 4	10	4.3
C7A (D)	10	4.6
C7A (L)	10	4.4
C7A*(D)	10	6.1
C7A*(L)	10	4.9

Note: C7A represents callus cultures in MS media supplemented with 8 mg/L NAA and 1.5 mg/L BAP and 3% sucrose. C7A* indicates in-vitro plants regenerated in MS medium supplemented with a combination of 1.0 mg/L BAP and 0.1 mg/L IBA. Dark and light conditions are represented by (D) and (L) respectively.

Graph 2: Shows the analysis of acemannan content in conventionally propagated and in vitro regenerated Aloe vera plants



Identity assessment of acemannan by ^{1}H-NMR

In 1998 Diehl and Teichmuller documented that the identity of acemannan in Aloe vera gel preparations can be significantly assessed by ^{1}H-NMR. Acemannan is a linear polysaccharide composed by β-(1→4)-linked mannan partially acetylated in positions 2, 3 or 6. These acetyl groups generate a characteristics signal (2.0-2.3 ppm) in ^{1}H-NMR, which shows the characteristic presence of acemannan and is considered as the fingerprint of acemannan in Aloe vera (Diehl and Teichmuller, 1998;). Similarly, the results obtained by the ^{1}H-NMR spectra of Aloe vera standard (Figure

4), plant gel (Figure 5) and callus extracts (Figure 6) confirm the presence of the immunomodulatory polysaccharide acemannan as shown below in Figure 3 (Bozzi et al., 2007; Krokida et al., 2011; Prerak et al., 2012; Ray et al., 2013).

Figure 3: [1]H- NMR spectra of Aloe vera gel powder concentrate 200:1 (Bozzi, et al., 2007)

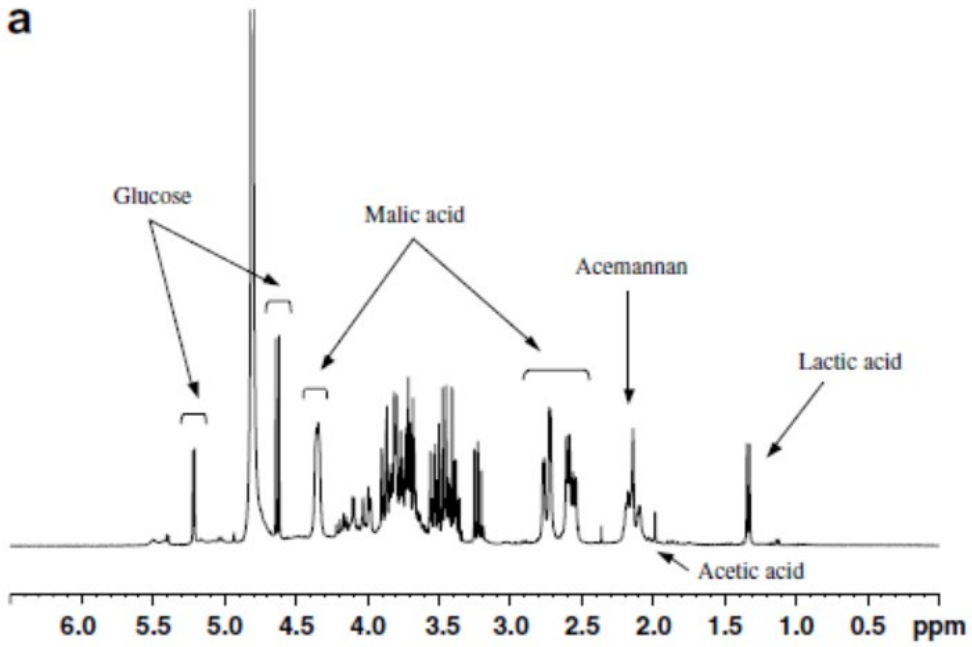


Figure 4: [1]H-NMR spectra of Aloe vera standard

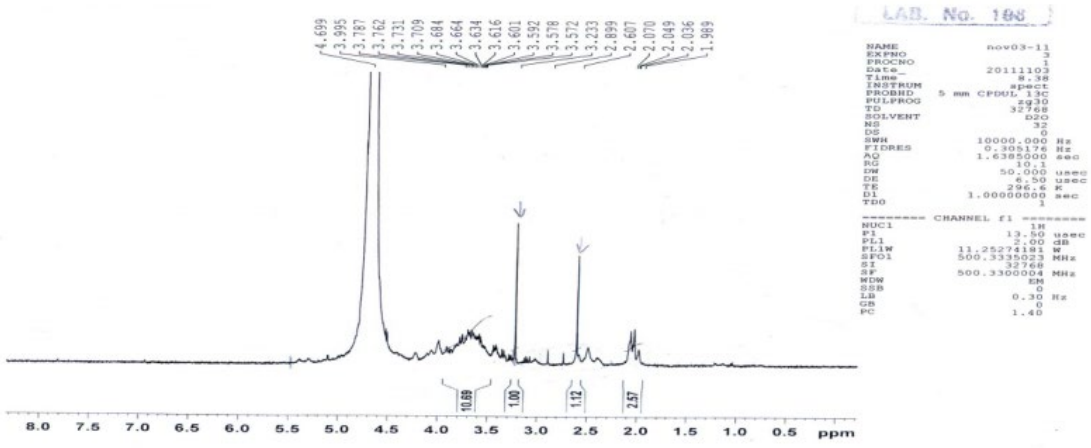


Figure 5: ^{1}H-NMR spectra of Aloe vera plant gel extract

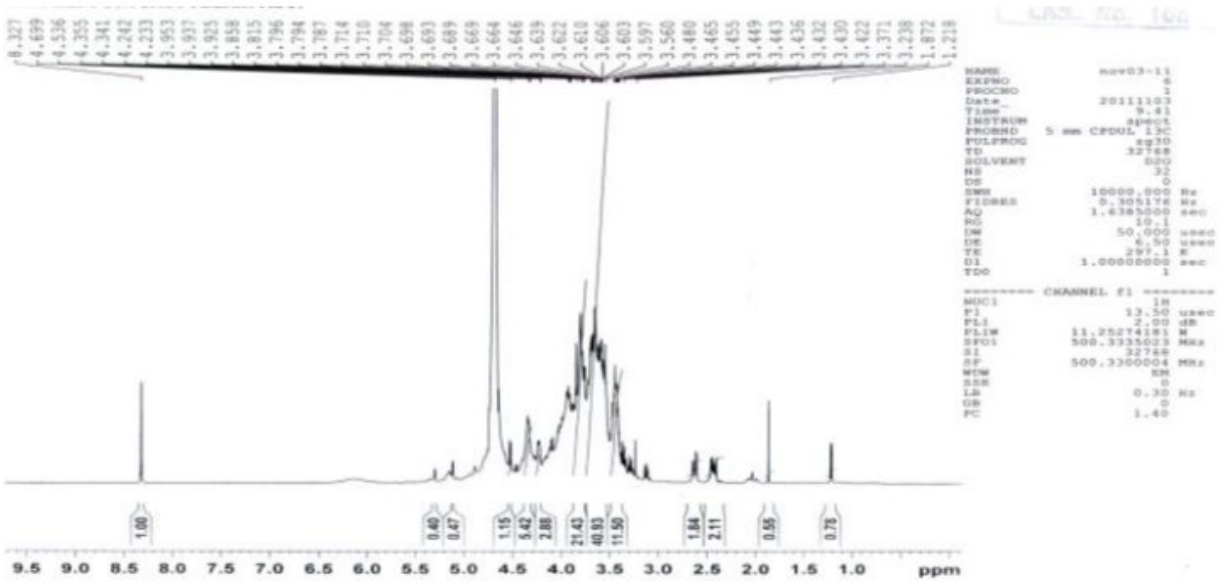
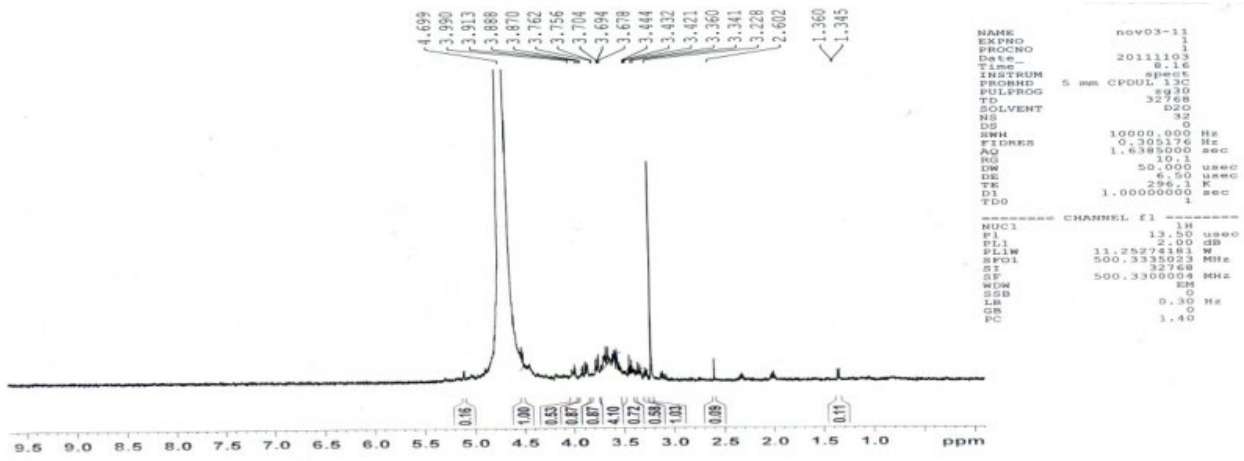


Figure 6: ^{1}H-NMR spectra of Aloe vera callus extract



Analysis of acemannan production and enhancement under different carbohydrate stress conditions

After optimization of several isolation and purification methods, protocols were optimized for qualitative and quantitative analysis of acemannan content in Aloe vera extracts. Acemannan production was confirmed in callus cultures by TLC analysis having Rf value of 0.6 which was similar to the standard compound. HPLC method was successfully employed and the quantitative analysis demonstrated the same retention time (2.0 min) when compared to the standard.

HPLC analysis provides strong evidence for the production and quantification of bioactive compounds. The extracts of callus cultures subjected to different carbohydrate stress treatments and kept under dark conditions for 30 days were analyzed for acemannan content by HPLC and strong variations in the acemannan levels detected can be seen in HPLC chromatograms. Of the different sugars and their concentrations tested, mannose and sucrose were found to be prominent in enhancing the production of this biologically potent polysaccharide.

The highest concentration of acemannan i.e 0.95 mg/mL was recorded in callus cultures incubated in media having 3% mannose sugar (Graph 4), which revealed a remarkable enhancement of acemannan production as compared to the in vivo propagation of Aloe vera plant in soil conditions having 0.43 mg/mL yield.

Calli cultured in media with 3% sucrose only increased minimal production of this compound in vitro giving 0.44 mg/mL yield (Graph 3). On the contrary, addition of glucose (Graph 5) and galactose (Graph 6) in the media proved to be least efficient and

suppressed the yield of acemannan in all the tested concentrations. Garro-Monge, et al., in 2008 reported acemannan detection by using high performance liquid chromatography (HPLC) in Aloe barbadensis Mill from inner gel and embryogenic calluses. According to their results the acemannan concentration in fresh leaves was much higher (85 mg/100 mL) than embryogenic calluses (0.8-2.1 mg/100 mL), when cultured on MS media having 3% sucrose and kept under dark.

Kim et al., in 2012 analyzed Aloe barbadensis suspension cultures for the production of extracellular polysaccharides and found a β-1,4- glucomannan, which is originally present in Aloe vera leaves. Under optimal conditions maximum levels of callus growth (20.4 g/L) was achieved in which the production of extracellular polysaccharide was 2.5 g/L.

Our experimental results showed a significant promoting effect of 3% mannose and 3% sucrose on callus cultured in dark conditions, stimulating acemannan concentration several folds (0.95 mg/mL and 0.44 mg/mL, respectively). Additionally, light also had a major effect on production of acemannan and the plants regenerated from callus in dark conditions cultured on MS media having 3% sucrose, showed higher levels (0.61 mg/mL) as compared to production in the in vitro plants under light conditions (0.49 mg/mL) and conventionally propagated Aloe vera plants in field (0.43 mg/mL). This effect was most probably due to the plant growth hormones and carbon source used in the culture media, suggesting utilization of tissue culture technology for higher production of acemannan.

According to investigations by Ferreira and his coworkers (2011), on the substances of soluble carbohydrates and starch of Dendrobium shoots. The upsurge in the sucrose concentration leads to the increase in the quantity of total soluble carbohydrates by almost six times greater in the dark than in light.

Man enzyme from fenugreek endosperm specially used GDP-Mannose as substrate and its incorporation was 300 times higher than GDP-Glucose for galactomannan biosynthesis (Fry,1988).

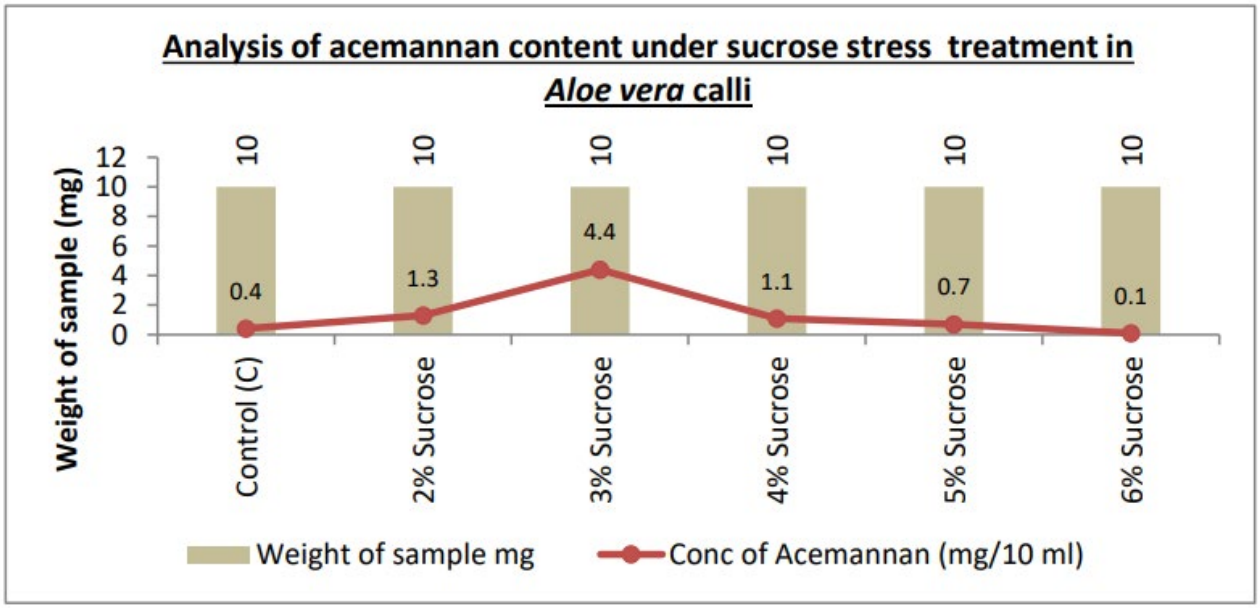
Another notable point in this experiment was the effect of light; acemannan was produced in much quantity in plants regenerated under dark conditions. According to Medina et. al. 1998, in Medicago strasseri plant callus cultures induced from leaves reported higher carbohydrate content in darkness than those obtained in light conditions.

And also the plants grown in absence of light don't photosynthesize. Hence the in vitro growth is mainly reliant on sugar included in the culture medium and the stocks available in the cultured tissues.

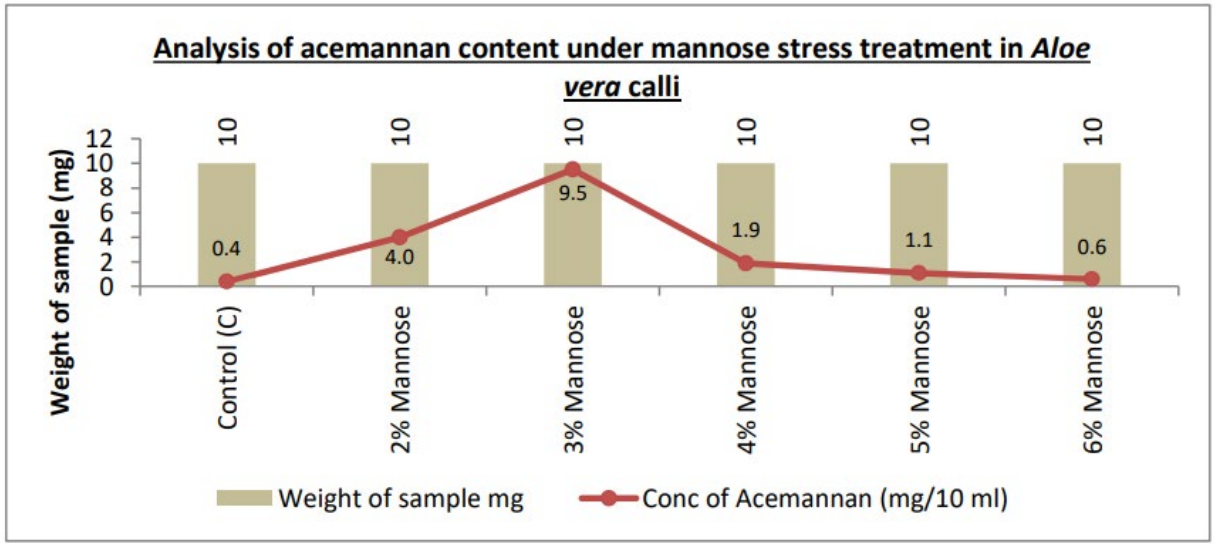
The reasons for these results need to be explored further and might include the high activity of the enzymes in the secondary metabolite pathway and carbon dioxide flux, suggesting the possibility as an alternative production method of Aloe vera bioactives.

The above findings pave the way for further studies on biosynthesis of medicinally important plant metabolites, and efficient multiplication of large number of plants from selected cell lines bearing high yield of specific active ingredient. This can be exploited for genetic biotransformation studies and further breeding programs. Consequently, intensified molecular and biochemical investigations on plant secondary pathways are needed to explore the future of plant cell cultures as producers of commercially useful compounds. The results of this research are very encouraging and will open up new vistas for future application and further advancement in plant biotechnology.

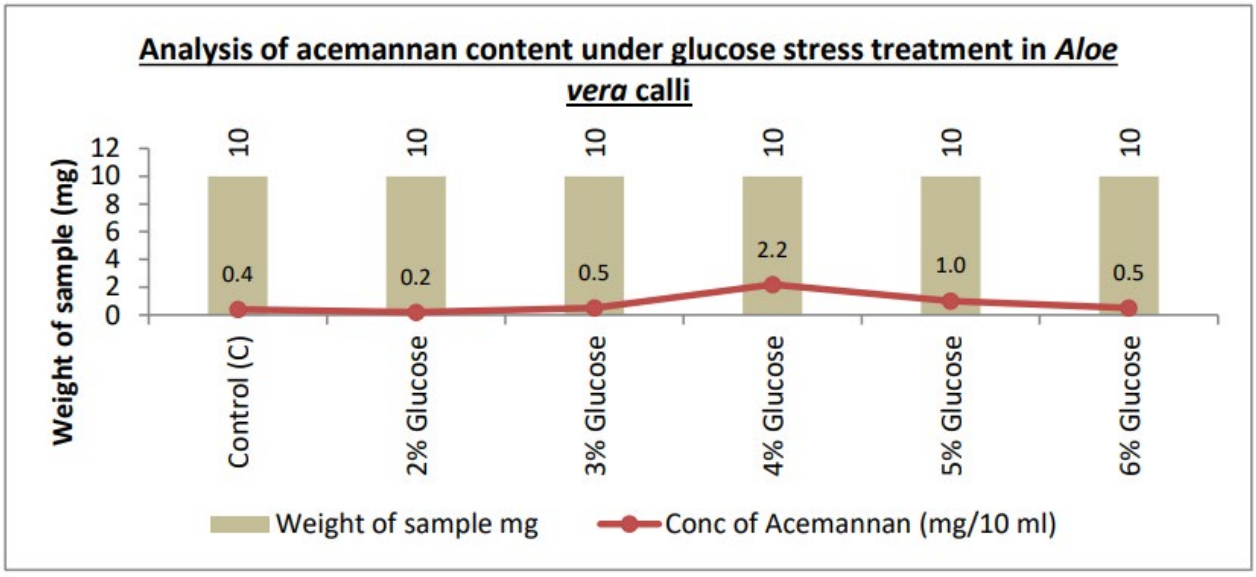
Graph 3: Shows the analysis of acemannan content under sucrose stress conditions in Aloe vera calli.



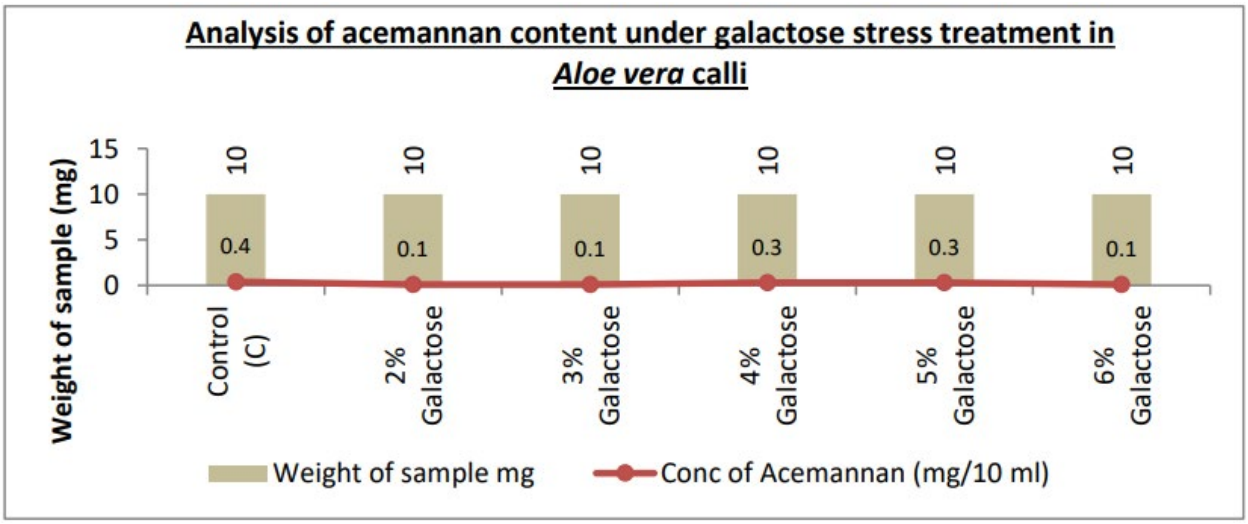
Graph 4: Shows the analysis of acemannan content under mannose stress conditions in Aloe vera calli.



Graph 5: Shows the analysis of acemannan content under glucose stress conditions in Aloe vera calli.



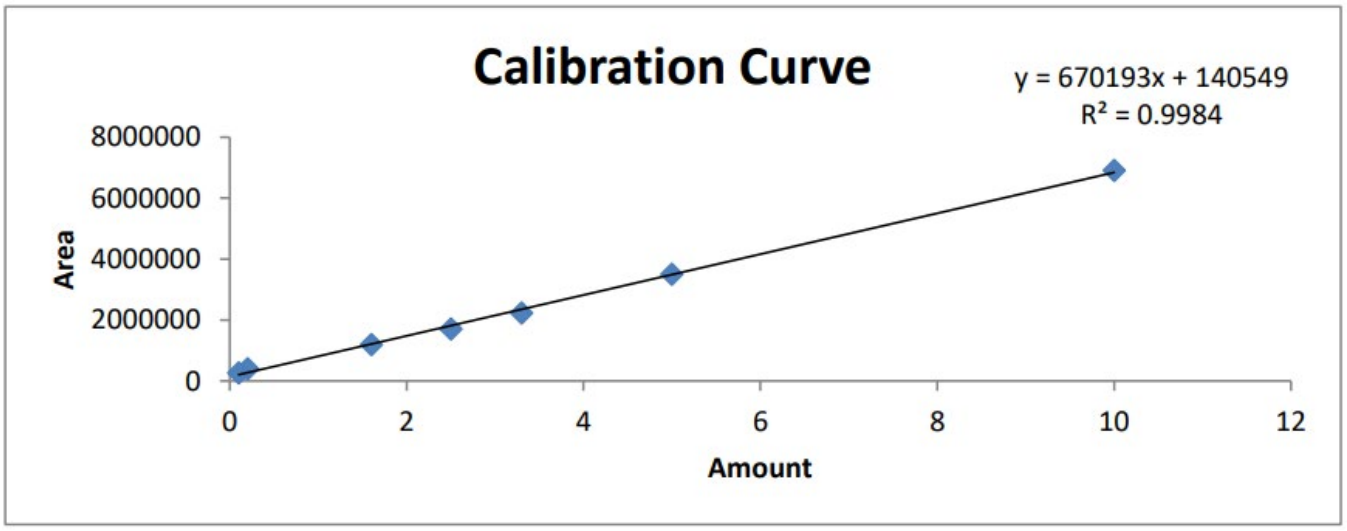
Graph 6: Shows the analysis of acemannan content under galactose stress conditions in Aloe vera calli.



Standard Calibration Plot

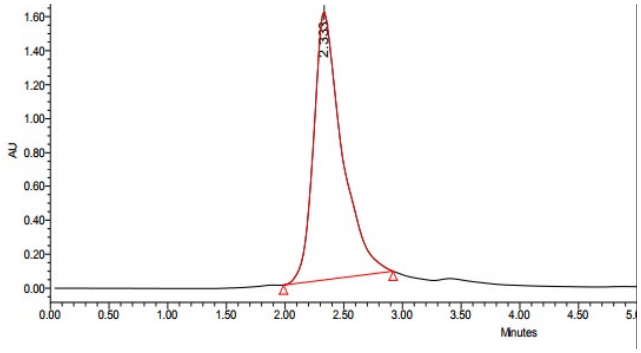
Quantification of Aloe vera acemannan was carried out at λ 209 nm. Linear calibration plot for acetyl-mannan was obtained at 10 different concentrations between 1-10 mg/10 mL. To check the reproducibility of results the data was obtained in triplicate. Linear regression analysis of the calibration plot yielded equations Y = 670193x + 140549. The calibration data indicated (r^2>0.9984) linearity of the detector response by HPLC-UV method. The results revealed excellent correlation between concentration and peak area (Graph 8). The linear calibration plot for acemannan obtained is shown below in Graph 8, which was then used to determine the concentration of acemannan in all the samples.

Graph 7: Shows the calibration curve of acemannan standard

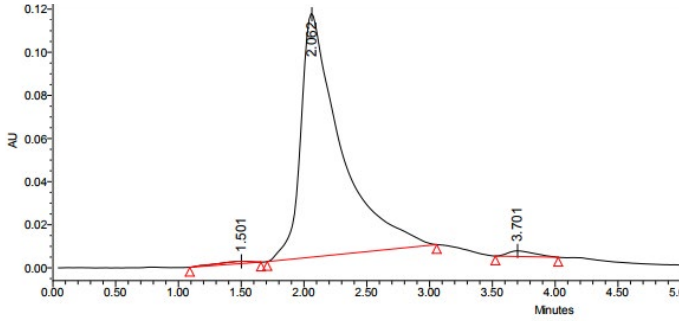


Graph 8: HPLC-UV chromatograms of Aloe vera standard, samples and control at λ 209 nm. The retention time of acemannan is at 2.1 min. The codes represented below indicate the Aloe vera varieties, in vitro grown Aloe barbadensis plants and callus cultures grown in MS medium supplemented with (2-6%) sucrose, mannose, glucose and galactose sugars.

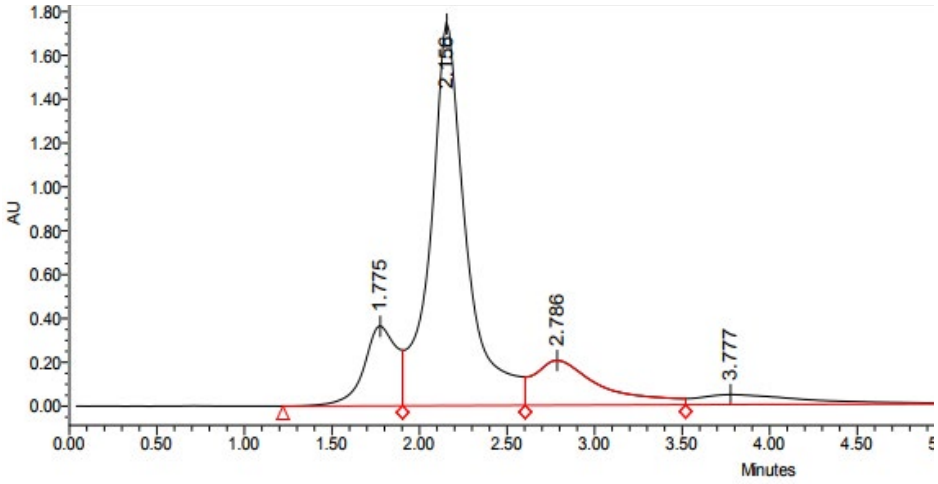
Acemannan Standard



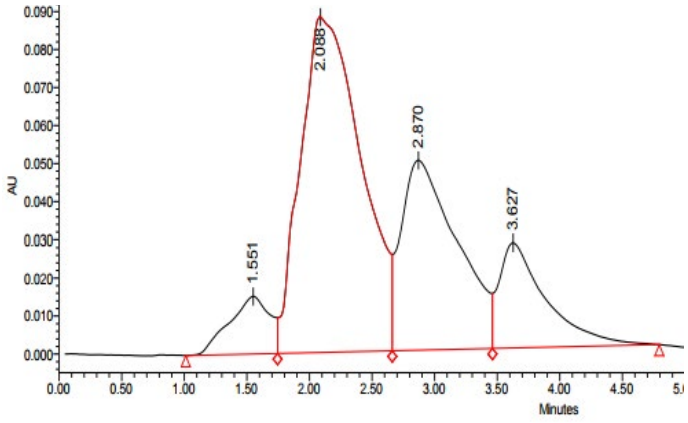
Control



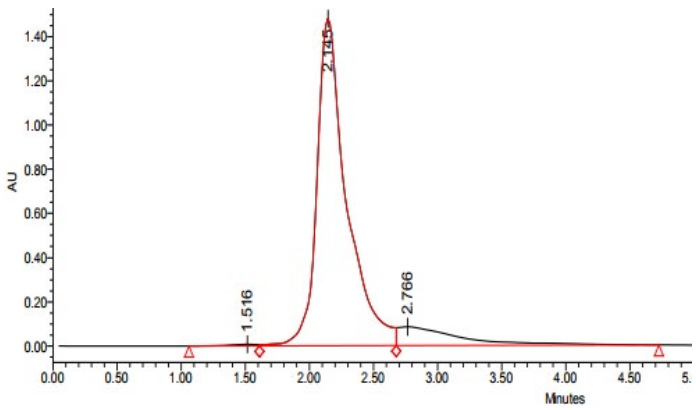
AC3S (L): Aloe barbadensis callus cultures under light conditions on 3% sucrose in culture media



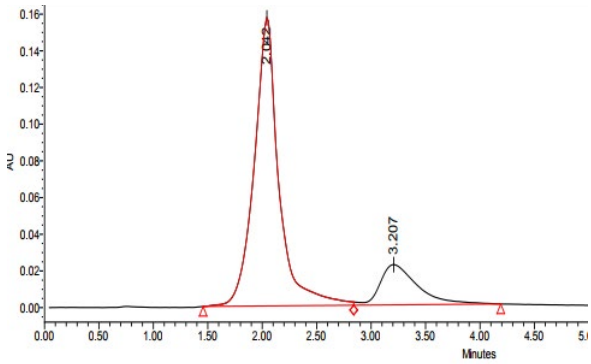
AV (1): Aloe vera variety 1



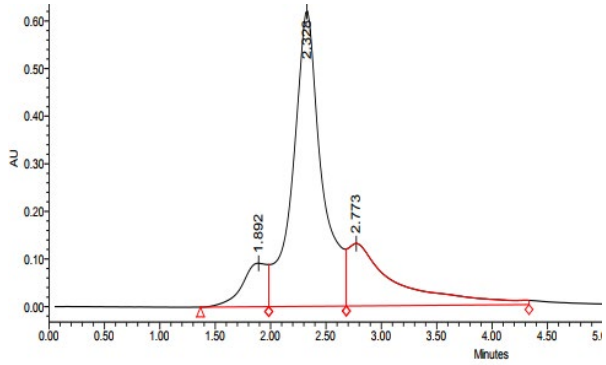
AV (2): Aloe vera variety 2



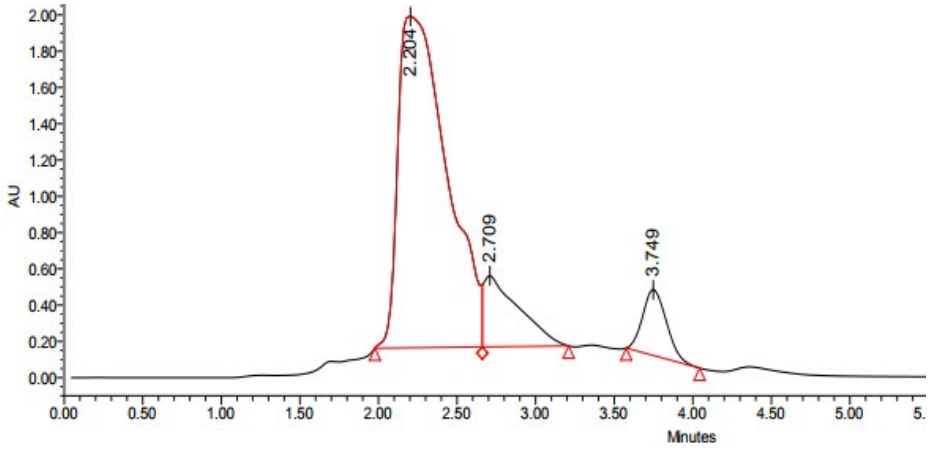
AV (3): Aloe vera variety 3



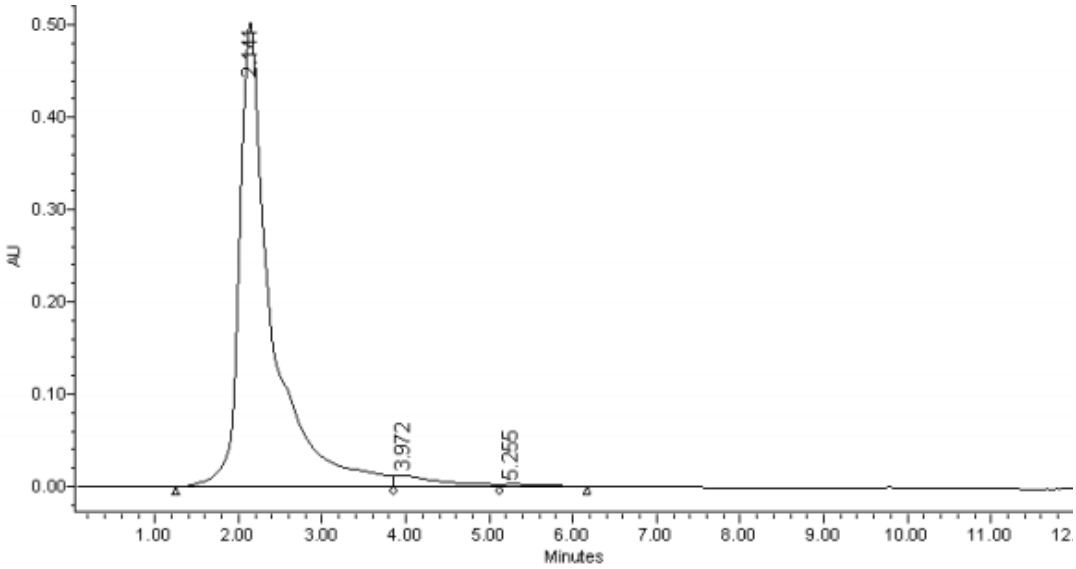
AV (4): Aloe vera variety 4



AVRG (D): In vitro regenerated Aloe vera plants under dark.

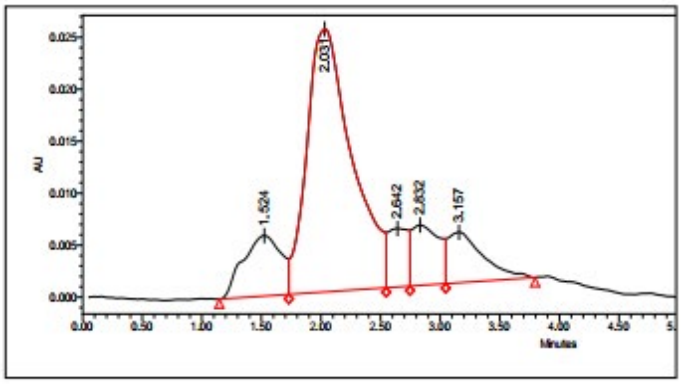


AVRG (L): In vitro regenerated Aloe vera plants under light.

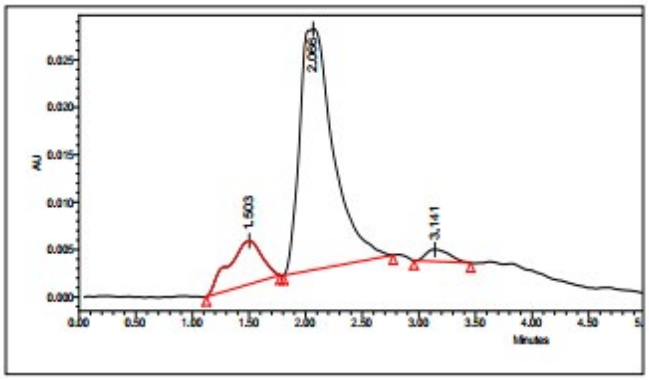


Aloe vera callus cultures grown in MS medium supplemented with (2-6%) Galactose

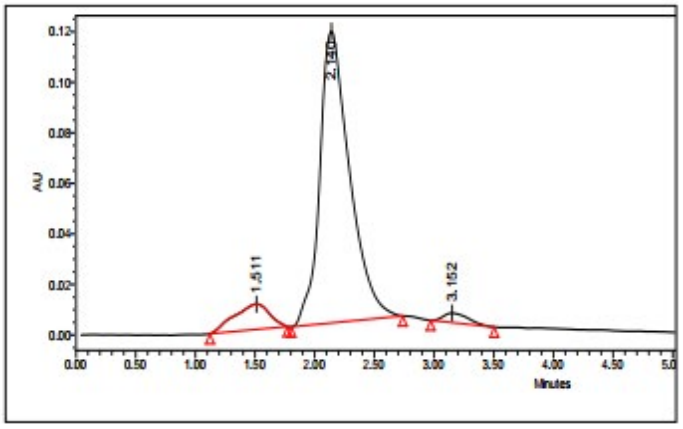
2% Galactose



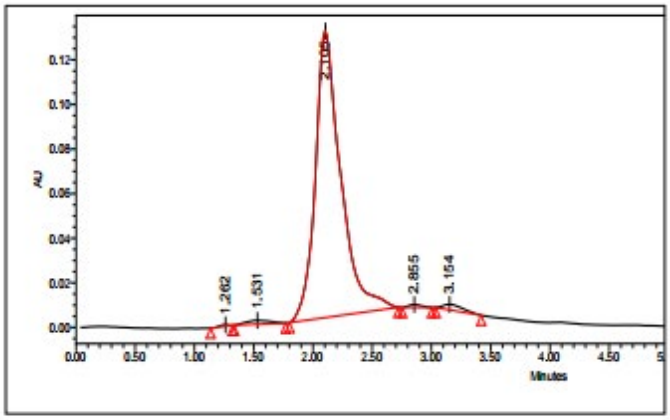
3% Galactose



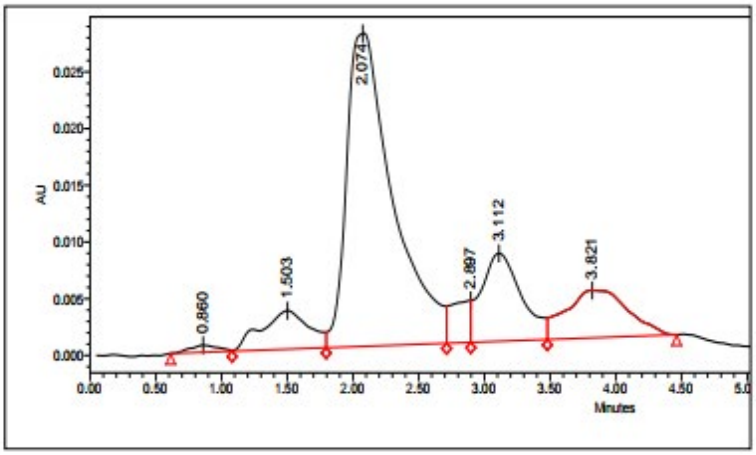
4% Galactose



5% Galactose

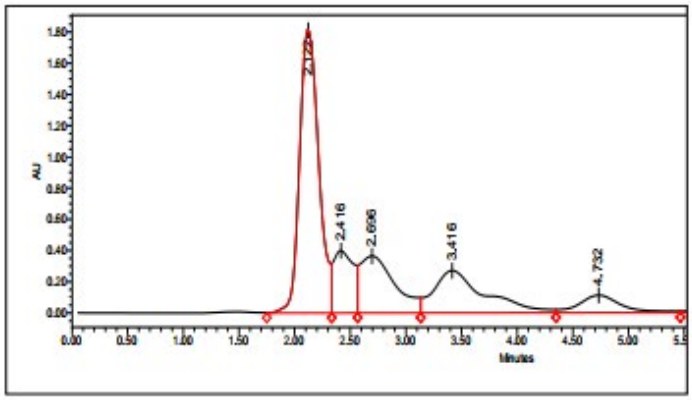


6% Galactose

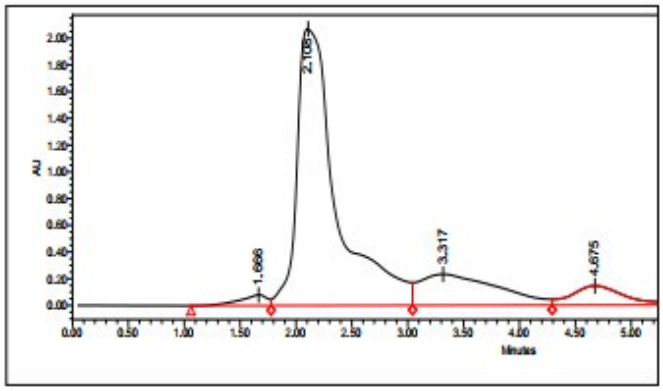


Aloe vera callus cultures grown in MS medium supplemented with (2-6%) Mannose

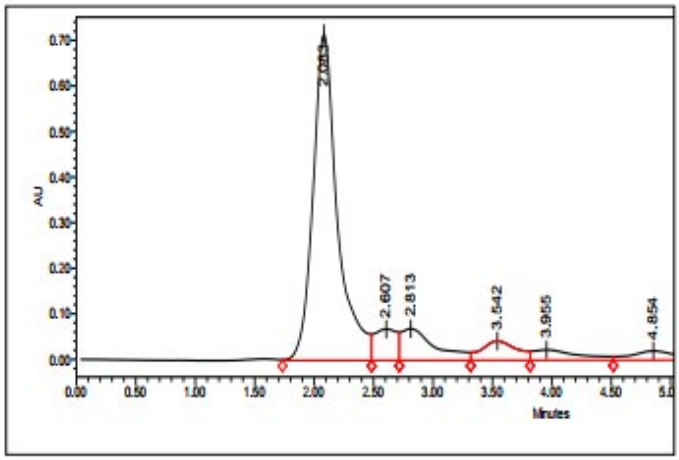
2% mannose



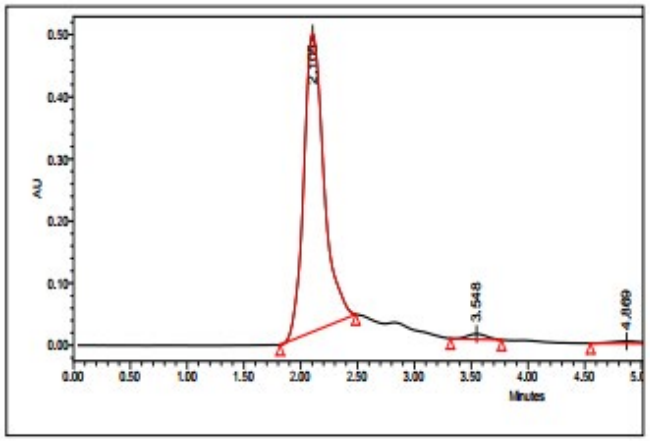
3% Mannose



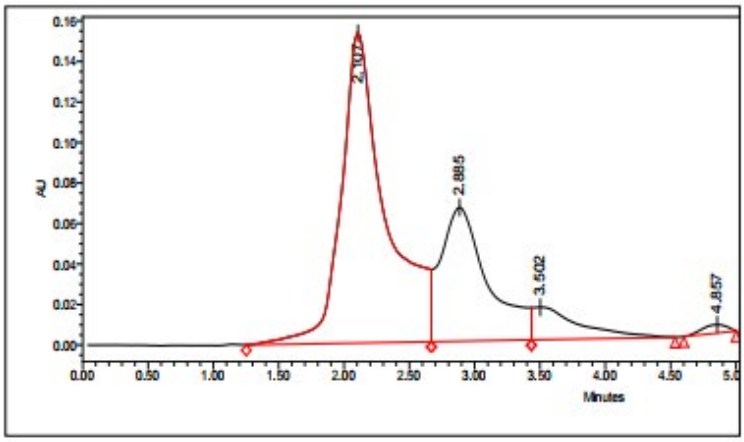
4% Mannose



5% Mannose

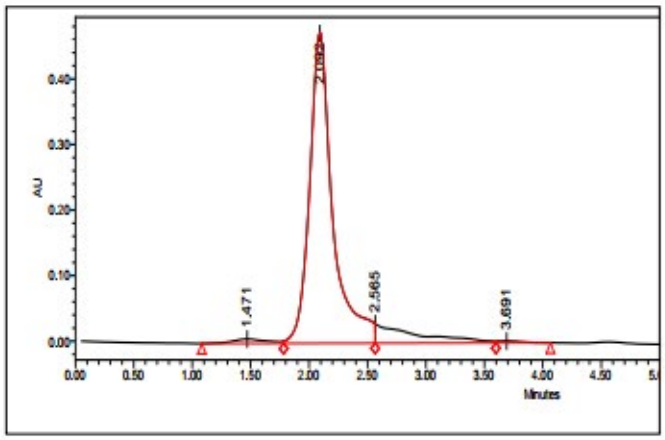


6% Mannose

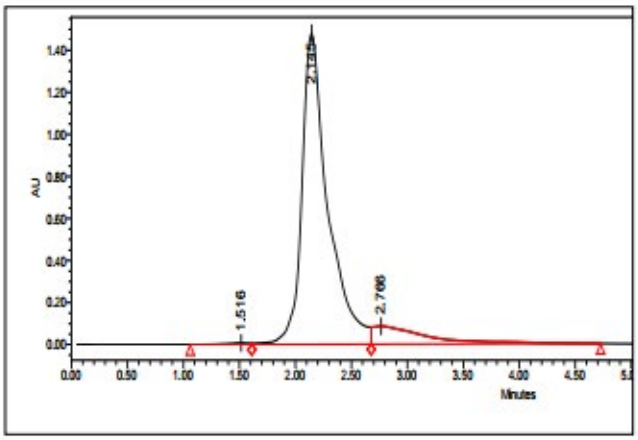


Aloe vera callus cultures grown in MS medium supplemented with (2-6%) Sucrose

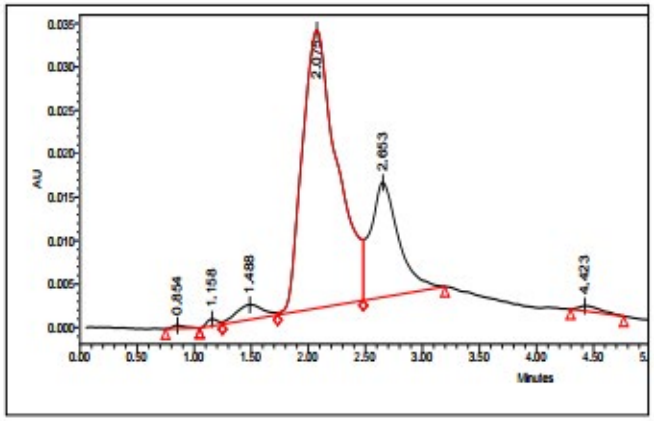
2% Sucrose



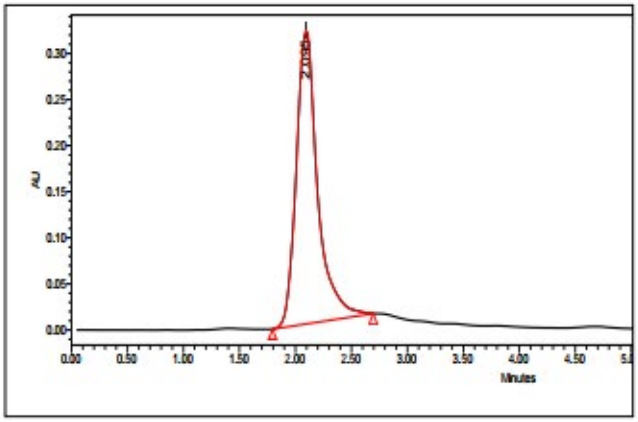
3% Sucrose



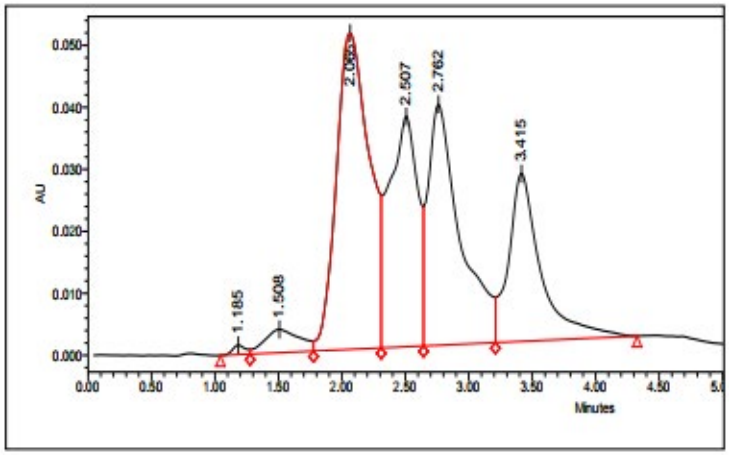
4% Sucrose



5% Sucrose

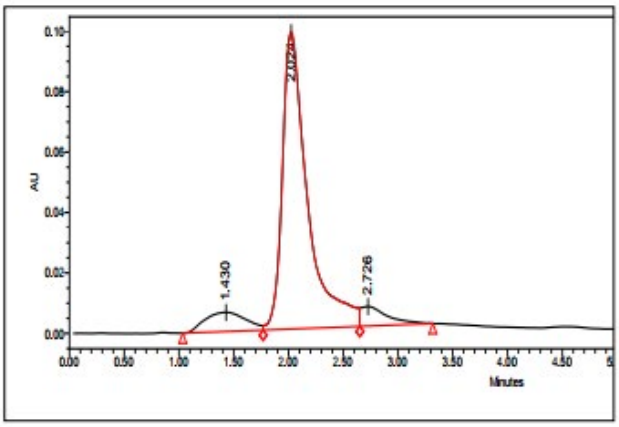


6% Sucrose

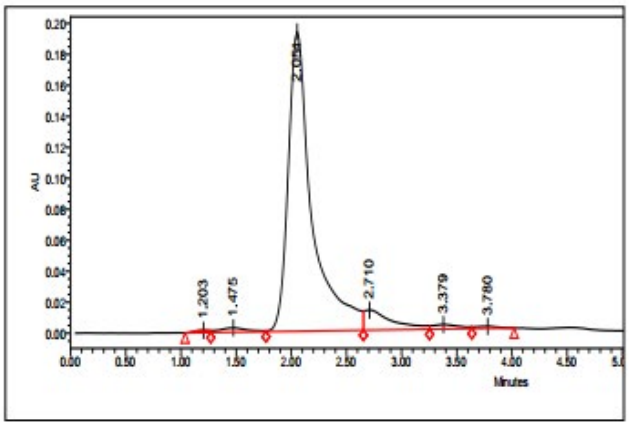


Aloe vera callus cultures grown in MS medium supplemented with (2-6%) Glucose

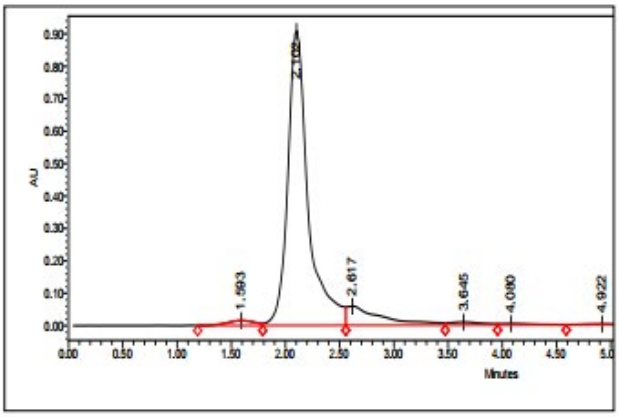
2% Glucose



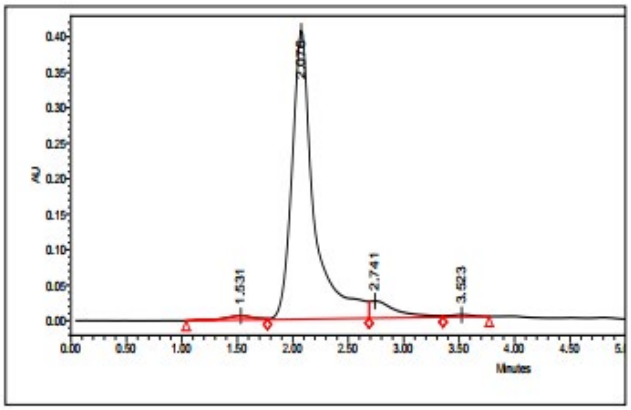
3% Glucose



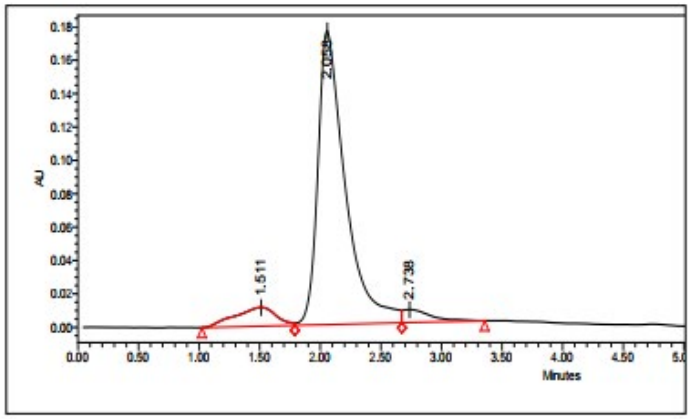
4% Glucose



5% Glucose



6% Glucose



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