International Journal of Pharmaceutics & Pharmacology

Available Online: https://ijpp.edwiserinternational.com

Evaluation of Antioxidant and Antibacterial Potential and α-Amylase Expression in the Leaf Callus Tissue of Rauvolfia serpentina (Linn.) Benth. Ex Kurz

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Article info

Received 07 April 2020
Revised 01 May 2020
Published 13 May 2020

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Abstract

Rauvolfia serpentina (Linn.) Benth. Ex Kurz belongs to the family Apocynaceae. The objective of the present study was to establish an effective protocol for regeneration of leaf explants from R. serpentina and to study α-amylase expression and phytochemical profiling by GC-MS (gas chromatography and mass spectrometry) technique. Further antibacterial activity was studied using the silver, gold and copper nano particles. The leaf explants were cultured on Murashige and Skoog (MS) medium containing Benzyl amino purine (BAP) (2.0 mg/L-1) and Naphthalene acetic acid (NAA) (1.0 mg/L-1) induced the formation of callus and expressed α-amylase. Explants growing on Murashige and Skoog medium fortified with 2,4-Dichlorophenoxy acetic acid (2,4-D) (1.0 mg/L-1), NAA (1.0 mg/L-1) with and without BAP (2.0 mg/L-1) and showed maximum concentration of protein on the 75th day. Silver, gold and copper nanoparticles were synthesized using the ethyl acetate extract of callus tissue and subjected to thin layer chromatography (TLC), which resolved 5 bands. These five bands were characterized by Fourier Transform Infrared (FTIR) Spectroscopy and screened for antimicrobial and antioxidant activities. TLC band 4 alone showed inhibitory activity against both gram positive and negative bacteria and potent antioxidant activity. GC-MS analysis revealed 21 compounds which included mainly phytosterols and fatty acids.

Keywords: MS medium, α-amylase, BAP, TLC, FTIR, GC-MS

Introduction

Rauwolfia serpentina (Linn.) Benth. Ex Kurz, popularly known as Sarpagandha, Indian snake root or devil pepper belongs to the family Apocynaceae. Under the genus Rauwolfia there are more than 100 species have been recorded, most of them are native to tropical and subtropical climatic zones which includes Central and South America, Australia Europe, Asia and Africa. *R. serpentina* are evergreen glabrous shrubs growing up to the height of 60 m and are commonly found in the moist, deciduous forests of Southeast Asia, including Bangladesh, Burma, Malaysia, India and Sri Lanka.

R. serpentina is widely used in Ayurveda, Unani, folk medicine and allopathic system. *R. serpentina* has been used since pre-Vedic period as a potential antidote for insect stings and snake bites [1], In folk and tribal medicine, the root extracts are used as laxative, uterine stimulant, diuretic, antihelmintic, antidote, expectorant and febrifuge [2-4]. Root extracts are used for the treatment of pneumonia, malaria, asthma, skin diseases, scabies, spleen diseases, eye diseases (opacity of cornea), circulatory disorders, AIDS, rheumatism, diarrhea and dysentery [2-6].

R. serpentina is a rich source of different varieties of chemical constituents. The root of this plant contains several alkaloids, which include ajmalicine, reserpine, serpentinine, ajmaline, yohimbine, ajmalimine, reserpiline, deserpidine, rescinnamidine, indobidine, rescinnamine, and serpentine [7]. Among the alkaloids, reserpine has attracted attention of researchers in the field of drug development throughout the globe. It is also useful in treating sedative insomnia, psychological disorders, excitement, epilepsy, traumas, anxiety, schizophrenia, insanity and in reducing blood pressure [4,5,8,9]. Reserpine exerts antihypertensive property by depleting the catecholamine [10,11]. Rescinnamine has the same activity as reserpine. however, it inhibits angiotensin-converting enzyme (ACE) that converts the angiotensin I, resulting in a decrease of plasma angiotensin II. Ajmaline possesses antiarrhythmic effect by blocking the sodium channel [10,12]. Serpentine has antipsychotic property because it inhibits type II topoisomerase. Yohimbine is selective alpha-adrenergic antagonist in blood vessels for the treatment of erectile dysfunction [12]. High concentration of phenols of *R. serpentina* revealed significant antidiabetic, hypolipidemic and antimicrobial properties. Flavonoids of *R. serpentina* help in preventing the oxidative cell damage and having anticancer, anti-inflammatory, and antioxidant properties [13-15]. The presence of saponins is responsible for the hemolytic activity and cholesterol binding property [16].

Ethnopharmacological studies have shown the antioxidant activity with respect to 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, reducing power and superoxide anion scavenging activity by the methanolic extract of leaves of *R. serpentina* [17]. Methanolic extract of *R. serpentina* rhizome also exhibited antioxidant activity as evident by the free radical scavenging activity and the increased level of superoxide dismutase, glutathione-S-transferase, catalase, glutathione peroxide, glutathione reductase, and decreased level of lipid peroxidation in CCl_4-induced hepatotoxicity rat model [18]. Ethanolic extract of root was shown to possess antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus*, *Bacillus subtilis* (Gram-positive) *Salmonella typhimurium* (Gram-negative bacteria) and *Klebsiella pneumoniae* [13,19,20]. Ethanolic extract of *R. serpentina* whole plant showed antivenom activity by neutralizing the toxic effect of *Naja naja* venom [21]. Aqueous ethanolic extract of the root of *R. serpentina* manifested hepatoprotective activity by protecting the liver from paracetamol-induced liver toxicity in rats [22]. This extract also has reversal effect on the levels of liver glycogen, serum bilirubin, thiobarbituric acid and glutathione and the activities of superoxide dismutase, catalase, glutathione peroxide, glutathione-S-transferase, glutathione reductase and Na^+K^+-ATPase [22]. Azmi et al. [6,9] reported the therapeutic potential of methanolic root extract in lowering the risk of atherogenic dyslipidemia, arteriosclerosis and glycosylation in alloxane-induced diabetic mice. Ezeigbo et al. studied the antidiarrheal property of methanolic extract of leaves of *R. serpentina* in castor oil-induced diarrhea in mice [23].

R. serpentina alkaloids have attracted worldwide attention in International markets for their high therapeutic efficiency and drug development. Indiscriminate collection of the plant, especially roots and overexploitation for commercial purposes have threatened this species with extinction. In order to conserve this valuable endangered species, an attempt has been made to define a method for *in vitro* propagation of this plant species and to study the phytochemical composition by gas chromatography and mass spectrometry (GC-MS) and α-amylase expression. Further, silver, gold and copper nanoparticles were synthesized using the ethyl acetate extract of callus tissue and subjected to thin layer chromatography (TLC). The TLC bands were then characterized by FTIR (Fourier Transform Infrared) Spectroscopy and screened for antimicrobial and antioxidant activities.

Materials and Methods

Source of explants of R. serpentina

Leaf segments of *R. serpentina* obtained from healthy mother plant (1-2 months old) (**Figure 1**) growing in the Kalasalingam University, Krishnankoil, Tamil Nadu, India, served as explants (**Figures 1a-d**).

Surface sterilization of explants

The collected pieces of leaves were washed under running water for 5-10 min to clean dust particles and then by liquid detergent (Vim soap oil). Subsequently, these explants were subjected to surface sterilization with (0.1%) mercuric chloride for 5 min and then was rinsed with distilled water five-six times.

Preparation of culture media and culture conditions

Tissue culture medium was prepared according to the method of Murashige and Skoog [24]. The medium contained 3% sucrose and solidified with 0.8% agar. The pH of the media was set to 5.8 and heat resistant growth regulators, Benzyl amino purine (BAP) and auxins like Naphthalene acetic acid (NAA) were added

to the medium and then sterilized for 15 min in an autoclave under 15 psi and 121°C.

Under laminar flow cabinet, disinfected leaves were aseptically excised and placed on the media in different orientations. The cultures were maintained at 27ºC with 8 h dark and 16 h light photoperiod per day with cool white fluorescent lights at an intensity of 85 µmol $m^{-2}s^{-1}$. This experiment was repeated thrice. Data on callus induction and growth were recorded periodically.

Investigation of callus development

The callus induction from the explants of *R. serpentina* was investigated on media containing cytokinins 2,4-D (2.0 mg/L^{-1}), BAP (2.0 mg/L^{-1}) and auxin NAA (0.1 mg/L^{-1}).

Preparation of protein and SDS-PAGE gel electrophoresis

Proteins extracts were prepared by homogenizing 500 mg of callus tissue (35 days old), mature leaf and root samples separately in Tris-HCl buffer (0.1 M, pH,8.0) at 4°C. The samples were sonicated by keeping it in an ice box. Homogenates were then centrifuged at 12,000 rpm at 4°C for 10 min. Protein concentration in the supernatant was determined by the method of Bradford using BSA as a standard [25].

Protein profiling was carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis **(**SDS-PAGE) method. Samples were denatured with Tris buffer (0.125 M, pH, 6.8), containing β-mercaptoethanol (5%), dithiothreitol (0.03%), glycerol (40%) and SDS (2%). The denatured protein (20 µl) was incubated in a water bath at 100°C for 3 min and loaded onto SDS-PAGE, which consisted of 12.5% separating gel and 5% stacking gel. Bromophenol blue (5 µl) was used as tracking dye. After the gel was cast, a volume of each of 15 µl protein samples from callus tissue, mature leaf and root samples were then loaded onto gels separately. A protein of known molecular weight marker standard (5 µl) (Bangalore Genei Pvt. Ltd.) was loaded in a separate lane adjacent to the sample wells. Electrophoresis was conducted at a constant current of 25 mA and a voltage of 150 V and until the bromophenol blue reached the bottom of the gel. After the run was over, the gels were carefully removed and immersed in a staining solution (0.5% Coomassie Brilliant Blue R-250) and destained in a solution containing 45% (v/v) methanol and 10% (v/v) acetic acid for 12 h. After proper destaining, the gel was documented and photographed. Molecular weight of the protein bands was determined by comparing the protein bands of molecular weight marker standards.

Western blot assay

For Western blotting, proteins that were resolved on SDS-PAGE on the basis of size were electrophoretically transferred onto nitrocelluose membranes (0.2 µm) (Millipore Corporation, USA). To block the nonspecific binding, the membranes were incubated with 5% (w/v) non-fat milk powder for 2 h. Membranes were probed with primary rabbit polyclonal anti-α-amylase (1:2000) antibody overnight. The membranes were then extensively washed and incubated for 2 h at room temperature with the horseradish peroxidase–conjugated secondary antibody. The bands were developed using ECL kit (Millipore, Bangalore, India). Protein expression levels were visualized with the Image Lab software (Bio-Rad, USA). Image densities of specific bands for α-amylase were normalized with the density of β-actin.

Preparation of callus tissue extracts

Thirty-five days old mature callus tissue was extracted with ethyl acetate for 8 h and concentrated using rotary evaporator and stored in a desiccator until use.

Synthesis and characterization of silver, gold and copper nanoparticles using callus tissue

Silver, gold and copper nanoparticles were synthesized by mixing 10 ml of ethyl acetate extract of callus tissue with 100 ml of aqueous solutions of silver, gold and copper nitrate separately at room temperature with constant stirring. The mixtures were heated at 60°C and then cooled to room temperature and kept in dark for 24 h. The mixture showed color change which was recorded visually. The surface morphologies and size of the silver, gold and copper nanoparticles were examined using Scanning Electron Microscopy (JSM-6360, JEOL), attached with Energy dispersive X-ray (EDX) diffractometer (Carl Zeiss, Germany).

TLC method

TLC is a method for separating the compounds from the mixture and determining the identity and purity of the compounds. In the present study, an aliquot of ethyl acetate extract of callus tissue was spotted on TLC silica gel plates (10 x 15 cm). The plates were developed using mobile phase of hexane and ethyl acetate in 8:2. After completion of the run, the plates were taken out from the development chamber, air dried and visualized under visible and UV light (240 and 300 nm). In the present study, the chromatogram revealed five distinct bands. The separated bands were marked and their retention factor (Rf) values were calculated and

recorded. The chromatogram was then photographed. The five bands of TLC chromatogram were scratched off separately, dissolved in alcohol, filtered, concentrated and then used for the antioxidant and antibacterial assays.

Antibacterial activity

TLC bioautographic method was exploited to study the antibacterial activity of Gram negative [*Escherichia coli* (MTCC 1652) and *B. licheniformis* (MTCC 73537)] and Gram positive [*Staphylococcus aureus* (MTCC 96) and *Pseudomonas aeroginasa* (MTCC 2453)] bacteria (NCCLS, 1993). All bacterial strains were provided from the microbiology laboratory of the Meenakshi Mission Hospital. All Bacterial strains were sub-cultured in nutrient agar broth for 24 h prior to testing.

In the present study, TLC chromatogram showed five bands. Each of the five bands of TLC was scratched off separately and was mixed with 5 ml of absolute ethanol and then allowed to stand for 10 min followed by filtration with Whatman No. 1 filter paper and then collected in glass vials. The recovered concentrates of each band were then tested for antibacterial activity by agar diffusion method.

About 0.1 ml of inoculum (1.5 x 10^8 /ml) of each bacterial strain was streaked out on molten Mueller Hinton agar plates with a sterile cotton swab. Wells of 7 mm diameter were made by scooping out agar with a sterile cork borer. The recovered concentrates of each of the five TLC bands were dissolved in 10% DMSO separately and loaded into the wells (200 µg/ well). A control well was added with 10% DMSO alone and served as negative control, while amphicilin (20 µg) was used as the positive control. Tests were carried out in triplicates and the plates were observed for the zone of inhibition and the diameter of the same was measured in cm. Further, antibacterial activity of three nanoparticles (gold, copper and silver) prepared using ethyl acetate extract of callus were also checked against two bacterial strains *S. aureus* (Gram positive) and *E. coli* (Gram negative) bacteria.

Determination of total antioxidant activity

Like antimicrobial activity, antioxidant activity was determined by TLC bioautographic method. Each of the five bands of TLC chromatogram was scratched off, mixed with absolute ethanol, filtered and concentrated. The recovered solutions of each band were then tested for total antioxidant activity [26]. Phosphomolybdenum method was exploited to assess the total antioxidant capacity. The assay is based on the reduction of molybdenum (VI) to green phosphate/molybdenum (V) complex at acidic pH by the sample analyte.

0.3 ml of aliquot from the recovered solutions of each TLC band was mixed with 1 ml of reagent solution containing sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM), incubated for 90 min at 95°C and then cooled to room temperature. Double beam spectrophotometer (UV-160 A, Shimadzu Corporation, Kyoto, Japan) was exploited to study the intensity of the developed green color at 695 nm against a blank. The total antioxidant activity was expressed in terms of the number of gram equivalent of ascorbic acid.

FTIR analysis

FTIR spectral analysis was performed by TLC bioautographic method using the recovered concentrates of five TLC bands obtained by using ethyl acetate extracts of 35-day old callus tissue. FTIR spectal analysis was performed in FTIR instrument (IRTRACER-100, Shimadzu, Japan) in the region of 4000 cm^{-1} to 500 cm^{-1} with PC based software and data processing. As mentioned earlier, each band of TLC was removed separately, mixed with absolute ethanol, filtered and concentrated. The recovered concentrates of each band were then encapsulated using KBr (100 mg) pellets in order to prepare translucent sample discs by applying pressure for FTIR analysis.

Phytochemical screening of ethyl acetate extract of callus tissue by GC-MS

The phytochemical screening was carried out in the ethyl acetate extract of 35-day old callus tissue by GC-MS technique. GC-MS analysis was carried out in an Agilent gas chromatography N6890 fitted with a HP-5MS fused silica column (5% phenyl methyl polysiloxane 30 m x 0.25 mm, film thickness 0.25 µm), interfaced with an Agilent 5975C VLMSD with triple axis mass detector. One microlitre of the sample was injected to the injected port. The oven temperature was raised from 40°C to 220ºC at a rate of 6ºC/min. The carrier gas was helium with a flow rate of 0.5 ml/min. Split ratio was 1:10, whereas split flow of 10 ml/min-1 mass range was 50 to 500. The sample was vaporized and then the various components of the sample was separated and analyzed. The MS was taken at 70 eV of ionization energy. GC-MS analysis produces a specific spectral peak for each component that get separated from the sample. GC-MS chromatogram was recorded on a chart electronically. The peak was measured from the base to the tip of the peak. The time elapsed between injection and elution is called the "retention time". Retention indices (RI) of the compounds were

determined by matching the spectra with reference spectra.

Identification of components

National Institute Standard and Technology (NIST), Version 14.0, Wiley 8.0 library database of NIST have more than 62000 patterns which are used for identifying chemical components. The unknown phytochemicals are identified by comparing their mass spectra with the spectrum of known compounds which are stored in the NIST library.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA). All the measurements are expressed as mean ± standard errors of means. A p value of < 0.05 was considered to be significant.

Results and Discussion

Induction of callus

The current study provided a protocol for large scale callus propagation of *R. serpentina* leaf explants. In the present study, during callus initiation, the explants did not show any leaching or browning of tissues. This indicates that the MS basal medium was the most effective for callusing of leaf explants.

A number of studies have shown that 2,4-D is inevitable and the choice of auxin for successful callus induction [27-29]. Later, it was found that addition of cytokinins is considered as the most important for enhancing the callus induction frequency. The choice of auxins and cytokinins and their concentrations play a significant role in determining the callus induction, growth and morphogenesis [30]. MS media without plant growth regulators failed to induce callus [31]. In the present study, a combination of BAP and NAA in the medium significantly influenced the induction of callus (**Figures 1a-f**). This is consistent with the report of Pandey et al. [27] who have reported higher frequency of callus formation when MS media was fortified with BAP and NAA. The above authors have also added that NAA as being the best plant growth regulator for producing green, compact and fast-growing *R. serpentina* callus [27]. Rashmi and Trivedi [32] studied the effects of different combinations and concentrations of plant growth regulators on callus growth and shown maximum callusing response (75% in stem and 77% in leaf) when MS media was supplemented with a combination of BAP (0.5 mg/L^{-1}) and NAA (1 mg/L^{-1}). Hence, in the present study, MS medium containing BAP at a concentration of 2 mg/L^{-1} and NAA at a concentration of 1 mg/L^{-1} was found to be optimal for the induction and growth of callus (**Figures 1a-f**).

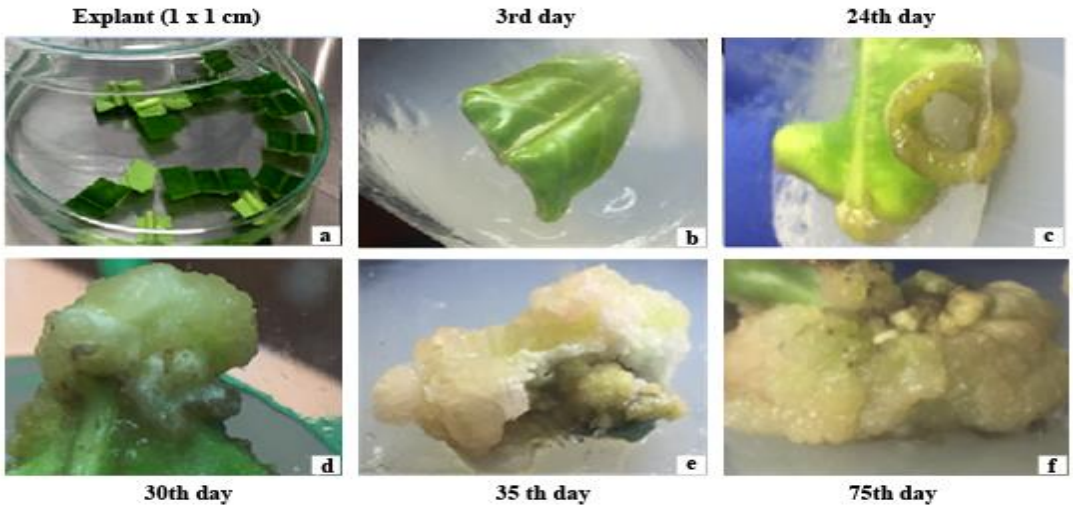

Figure 1: Different stages of callus development in MS media supplemented with BAP (2.0 mg/L^{-1}) and NAA (0.1 mg/L^{-1}).

Studies on a-amylase expression in callus tissue, mature leaves and roots by SDS-PAGE

SDS-PAGE is the most widely used analytical method to resolve components of a protein mixture. In the present study, the total protein was estimated from mature cream-colored callus, mature leaves, and roots.

Protein profiling of mature leaves, root and leaf callus by SDS-PAGE resolved around 16 bands ranging from 2 to 240 KDa (**Figures 2a**). Protein profiles further showed variability in the number of bands, band pattern and band intensity. Out of 16 protein bands, molecular weights 5 to 240 kDa have shown the same pattern of protein banding in leaf callus, mature leaves and root samples. The callus tissue revealed 4 bands at 50, 40, 18, and 13 kDa. The root sample also showed similar protein bands with varying intensities. However, all the four bands were found to be absent in mature leaf samples. From these observations, it is inferred that callus showed the highest number of protein bands followed by root. 50 kDa protein found predominantly in both callus and root confirms the α-amylase enzyme expression by comparison with reference sample. This was further confirmed by Western blotting (**Figures 2b**).

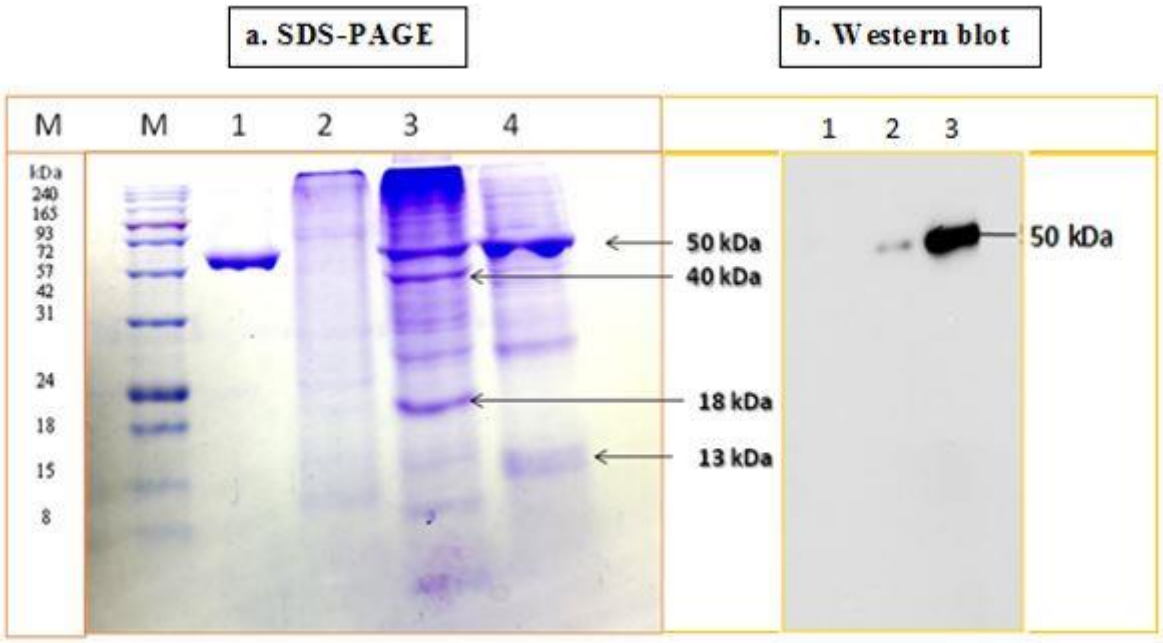

Figure 2: Separation of protens by SDS-PAGE and amylase expression by Western blot.

Figure 2a Separation of protein samples run on 12% SDS-PAGE (M-Marker, 1-α-amylase alone, 2-crude leaf protein, 3-crude leaf callus protein, and 4-crude root protein) 2b. Western blot assay (1-leaf protein, 2-leaf callus protein, and 3-Root protein).

Further, the protein content was determined in normal leaf, root as well as in the callus grown on media supplemented with a combination of NAA (0.1 mg/L^{-1}) and 2,4-D (2.0 mg/L^{-1}) with and without BAP (2.0 mg/L^{-1}) at various time points namely 3rd 24th, 30th, 40th and 75th day (**Table 1**). The callus grown on medium containing only NAA (0.1 mg/L^{-1}) and 2,4-D (2.0 mg/L^{-1}) showed a steady increase in protein content on 3rd, 24th, 30th days. Thereafter, the protein content declined. Whereas the protein content observed in the callus grown on medium fortified with NAA (0.1 mg/L^{-1}) + 2,4-D (2.0 mg/L^{-1}) and BAP (2.0 mg/L^{-1}) showed maximal increase on the 30th day and this level was maintained in 40 and 75 day old callus (**Table 1**). The observed increase in the protein levels on, 3rd, 24th and 30th days may be attributed to the mitotic activity occurring during the exponential and linear growth phases. This reduction in protein levels after 30 days may possibly be occurred due to the differentiation phase.

Characterisation of silver and gold nanoparticles

SEM analysis revealed that silver nanoparticles synthesized through green chemistry are well dispersed and spherical in shape (**Figures 4a and b**). **Figure 5** shows the SEM images of the gold nanoparticles. SEM images showed that most of the gold nanoparticles are highly homogenous and predominately spherical in shape having smooth surface (**Figure 5a**). EDX analysis revealed the presence of silver and gold elements, confirming the successful synthesis of gold and silver nanoparticles (**Figures 4c and 5c**).

Table 1: Effects of 2,4-D and NAA with and without BAP on protein concentrations at different time points during callus development.

S. No.	Time points	Protein concentration (mg)	
		Auxin and cytokinin	
		NAA (0.1mg/L^{-1}) + 2,4,D (2 mg/L^{-1})	NAA (0.1 mg/L^{-1}) + 2,4,D (2 mg/L^{-1}) + BAP (2 mg/L^{-1})
1	3rd day	1.663 ± 0.10	1.997 ± 1.21
2	24th day	3.666 ± 0.04 [a]	2.909 ± 0.01
3	30th day	4.000 ± 0.03[a]	6.612 ± 0.05 [a.b]
4	40th day	3.212 ± 0.78	6.907 ± 0.56 [a.b]
5	75th day	3.091 ± 1.00	6.999 ± 0.08 [a.b]

Values are expressed as mean ± SEM; a – Compared to 3rd day; b – Compared to 24th day; c – Compared to 30th day; d– Compared to 40th day; Significant at p< 0.005

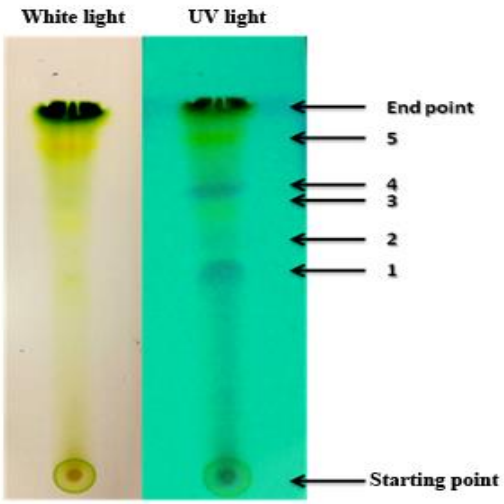

Figure 3: TLC chromatogram showing bands under white light and UV light.

Antibacterial activity using agar well diffusion method

Antibacterial activity was evaluated using the recovered concentrates of five TLC bands eluted using the ethyl acetate extracts of 35-day old callus tissue. Antibacterial activity was tested against Gram negative *E. coli* and *P.aeroginosa* and Gram positive bacteria *S. aureus* and *B.licheniformis* by agar well diffusion method. If the sample examined had antimicrobial activity, a clear zone would be formed on the surface of the agar, representing an inhibition of bacterial growth. In the present study, TLC chromatogram revealed five bands (**Figure 3**) Interestingly, of the five bands of TLC tested, 4th band or fragment alone has shown pronounced antibacterial activity with maximum inhibition zone of diameter 1.7 cm against gram positive bacteria and the lowest inhibition zone of diameter 1.2 cm in gram negative bacteria. The other four concentrates of TLC bands, (TLC band-1, TLC band-2, TLC band-3 and TLC band-5) were found to be ineffective against all the tested bacteria (**Figure 6**) Nevertheless, the positive effect observed with the concentrate of TLC band-4 indicates that the *R. serpentina* callus synthesizes compounds responsible for antibacterial activity. This is in fair correlation with a number of earlier studies, which have shown good antibacterial activity in the leaf, shoot and root extracts of *R. sepentina* [19,20]. The antibacterial effects of three nanoparticles (gold, copper and silver) using ethyl acetate extract of callus tested against Gram positive bacteria (*S. aureus*) and Gram negative (*E. coli*) are presented in **Figure 7**. Among the three nanoparticles, silver nanoparticles alone recorded antibacterial activity against both Gram positive (*S.aureus*) and Gram negative bacteria (*E. coli*).

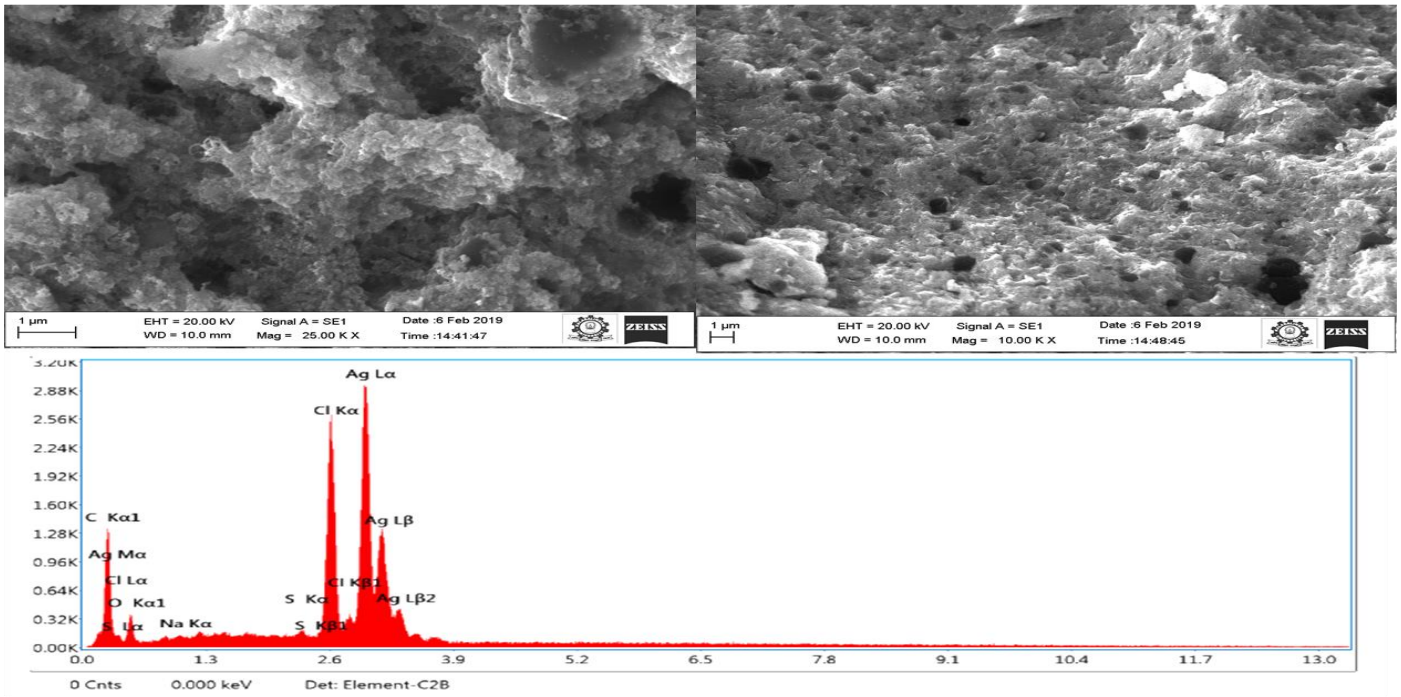

Figure 4: SEM images with EDX analysis of silver nanoparticles synthesized using ethyl acetate extract of callus of *R. serpentine.*

Scanning Electron microscopy images (a and b) represents silver nanoparticles synthesised using ethyl acetate extract of callus of R. serpentina. Figure 4c represents the Energy-Dispersive X-ray microanalysis of silver nanoparticles.

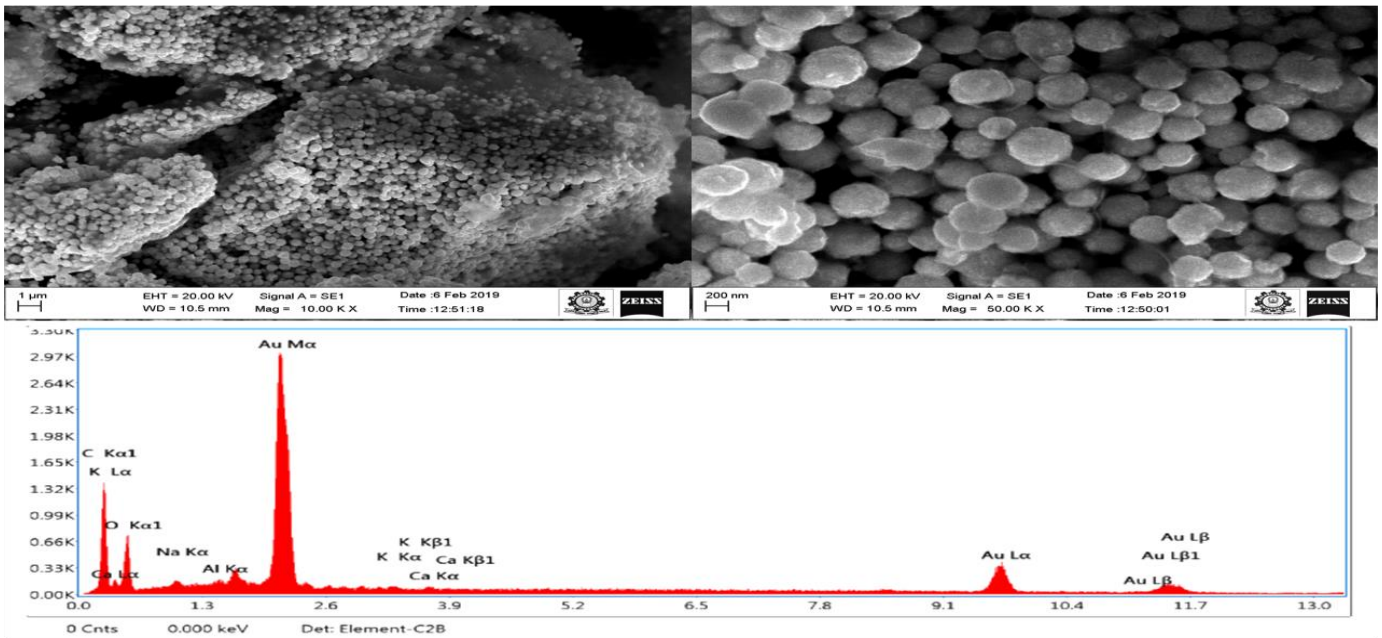

Figure 5: SEM images with EDX analysis of gold nanoparticles synthesized using ethyl acetate extract of callus of *R. serpentine.*

Figure 5 Characterisation of gold nanoparticles. Scanning Electron microscopy images (a and b) represents gold nanoparticles synthesised using ethyl acetate extract of callus of R. serpentina. Figure 5c represents the Energy-Dispersive X-ray microanalysis of gold nanoparticles.

Nevertheless, comparatively, maximum inhibition with silver nanoparticles was witnessed against Gram negative bacteria (*E. coli*) (3.7 cm), while the activity was moderate against Gram positive bacteria (*S. aureus*) (1.5 cm). This observation indicates that the Gram negative bacteria, *E. coli* was more sensitive than the Gram positive *S.aureus.* This finding is consistent with the report of Murthy and Narayanappa [20], who have shown maximum antibacterial activity of leaf or root extracts of *R. sepentina* for Gram negative bacteria than Gram positive bacteria.

Antibacterial activity using agar well diffusion method

Antibacterial activity was evaluated using the recovered concentrates of five TLC bands eluted using the ethyl acetate extracts of 35-day old callus tissue. Antibacterial activity was tested against Gram negative *E. coli* and *P. aeroginosa* and Gram-positive bacteria *S. aureus* and *B. licheniformis* by agar well diffusion method. If the sample examined had antimicrobial activity, a clear zone would be formed on the surface of the agar, representing an inhibition of bacterial growth. In the present study, TLC chromatogram revealed five bands (**Figure 3**) Interestingly, of the five bands of TLC tested, 4th band or fragment alone has shown pronounced antibacterial activity with maximum inhibition zone of diameter 1.7 cm against gram positive bacteria and the lowest inhibition zone of diameter 1.2 cm in gram negative bacteria. The other four concentrates of TLC bands, (TLC band-1, TLC band-2, TLC band-3 and TLC band-5) were found to be ineffective against all the tested bacteria (**Figure 6**) Nevertheless, the positive effect observed with the concentrate of TLC band-4 indicates that the *R. serpentina* callus synthesizes compounds responsible for antibacterial activity. This is in fair correlation with a number of earlier studies, which have shown good antibacterial activity in the leaf, shoot and root extracts of *R. sepentina* [19,20]. The antibacterial effects of three nanoparticles (gold, copper and silver) using ethyl acetate extract of callus tested against Gram positive bacteria (*S. aureus*) and Gram negative (*E. coli*) are presented in **Figure 7**. Among the three nanoparticles, silver nanoparticles alone recorded antibacterial activity against both Gram positive (*S. aureus*) and Gram-negative bacteria (*E. coli*). Nevertheless, comparatively, maximum inhibition with silver nanoparticles was witnessed against Gram negative bacteria (*E. coli*) (3.7 cm), while the activity was moderate against Gram positive bacteria (*S. aureus*) (1.5 cm). This observation indicates that the Gram-negative bacteria, *E. coli* was more sensitive than the Gram positive *S. aureus.* This finding is consistent with the report of Murthy and Narayanappa [20], who have shown maximum antibacterial activity of leaf or root extracts of *R. sepentina* for Gram negative bacteria than Gram positive bacteria.

Antioxidant activity

The total antioxidant activity of recovered concentrates of five TLC bands obtained using ethyl acetate extracts of 35-day old callus tissue was evaluated by comparing reference compound, ascorbic acid. Of the 5 TLC compounds tested, 4th band expressed the highest antioxidant activity and was more than the ascorbic acid (**Figure 8**).

FTIR analysis using TLC products

In the present study, FTIR spectra was obtained from the recovered concentrates of five TLC bands obtained by using ethyl acetate extracts of 35-day old callus tissue. TLC band pattern in normal and UV light are shown in **Figure 3**. The functional groups were identified by comparing the peak values in the IR spectra with that of the reference compounds. The FTIR spectral data of both control (silica gel alone) and the 5 bands obtained on the TLC using ethyl acetate extract of *R. serpentina* callus tissue are presented in **Figures 9a-f**.

The samples were analyzed in the spectral region of 500 to 4000 cm^{-1}. All the five bands of TLC exhibited a characteristic absorption maximum at 2386 cm^{-1}, indicating the presence of C-H stretching. Another characteristic absorption maxima at 1095.57 cm^{-1}, indicating the presence of C-O) for a hydroxyl (-OH), which was observed in all the TLC bands, except band-3. TLC bands-3 and 5 revealed another characteristic peak at 1540-1560 cm^{-1}, which is indicative of C=O aromatic stretch. Besides, the five TLC bands showed the absorption maxima at 3000-4000 cm^{-1}, indicating the presence of hydroxyl groups, which includes H-bonded OH stretch, polymeric OH stretch, dimeric OH stretch and nonbonded hydroxyl group of primary, secondary, tertiary alcohol and phenol. The absorption maxima at 2882 cm^{-1} and 2380 cm^{-1} (for C-H stretching), at 1641 cm^{-1} (C=C stretching), 974 cm^{-1} (C-H bending of aromatic hydrocarbons) and 798 cm^{-1} (aromatic carbons) (**Table 2**).

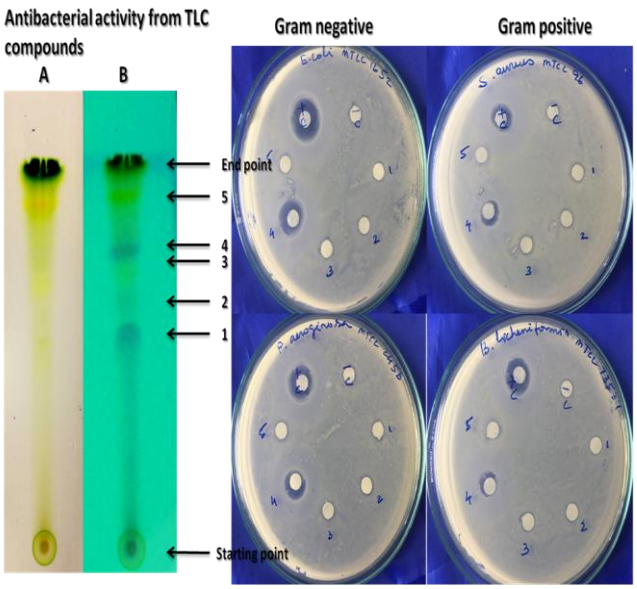

Figure 6: Antibacterial activity by TLC bioautographic method.

Figure 6 shows antibacterial activity by TLC bioautographic method; TLC chromatogram showing five bands. Antibacterial activity was checked using the concentrates of five TLC bands against Gram negative (a) E.coli (MTCC 1652), (b) P.aeroginasa (MTCC 2453) and Gram positive bacteria (c) S.aureus (MTCC 96) and (d) B.licheniformis (MTCC 73537).

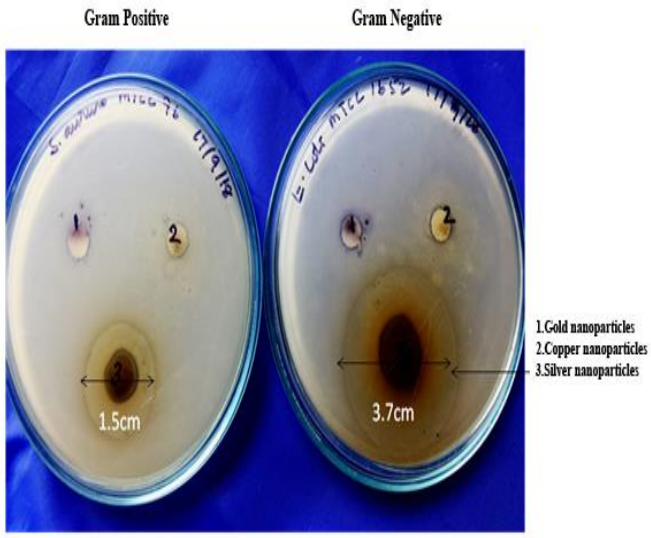

Figure 7: Antibacterial activity of gold, copper and silver nanoparticles using ethyl acetate extract of callus tissue of *R. serpentine*.

Figure 7 shows antibacterial activity of gold, copper and silver nanoparticles using ethyl acetate extract of callus tissue of R. serpentina. Antibacterial activity shown against Gram positive S. aureus (MTCC 96) and Gram-negative E. coli (MTCC 1652).

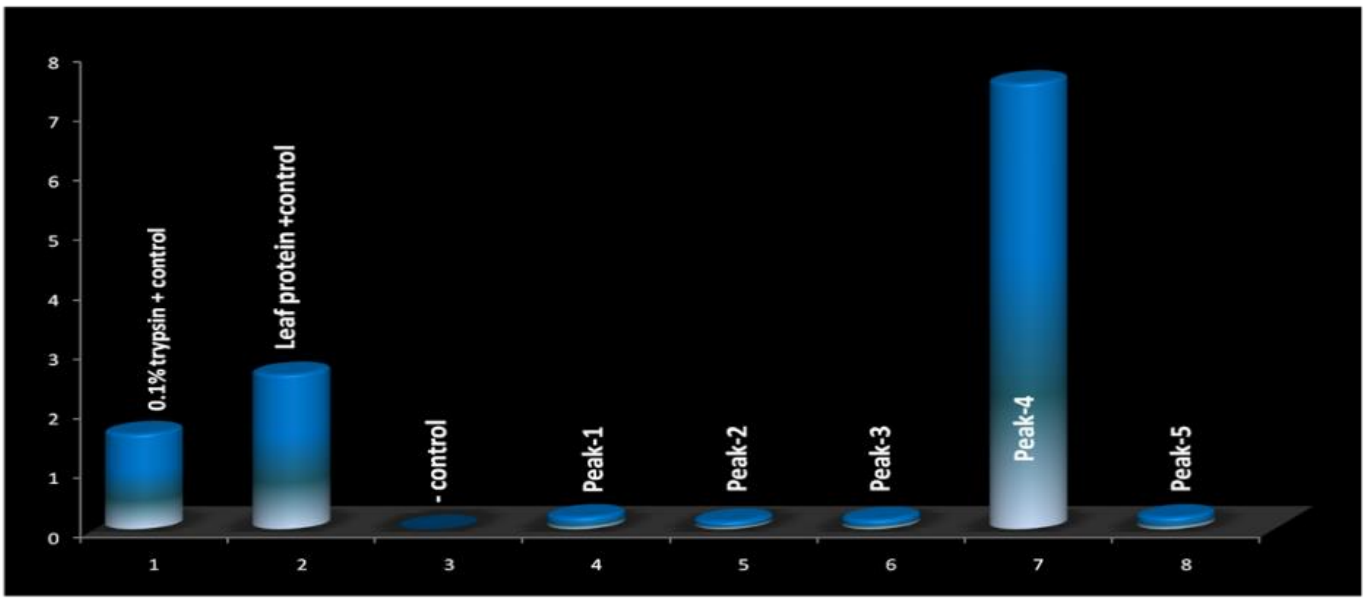

Figure 8: *Antioxidant activity by TLC bioautographic method using ethyl acetate extract of callus tissue of R. serpentine. Values are expressed as mean ± SEM; a – Compared to Control; b – Compared to ascorbic acid; Significant at p < 0.005*

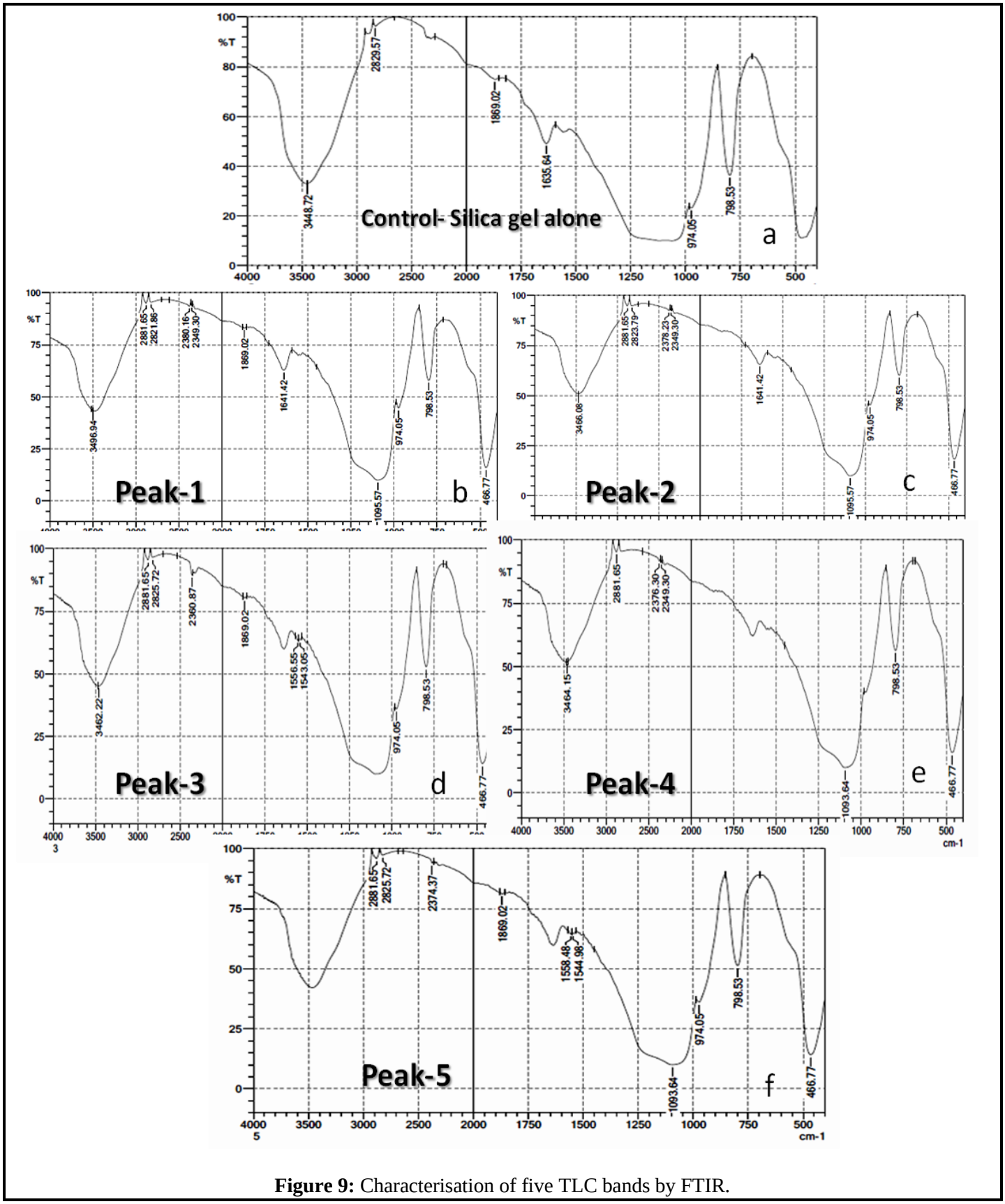

Figure 9: Characterisation of five TLC bands by FTIR.

Table 2: FTIR spectral peak values and functional groups obtained for the TLC bands resolved from the ethyl acetate extract of leaf callus of *R. serpentine.*

Silica gel	TLC Band-1	TLC Band-2	TLC Band-3	TLC Band-4	TLC Band-5	Functional groups	
Wave numbers cm^{-1}							
3448.72	3495.94	3485.08	3462.22	3464.15	--	O-H	Hydrogen bonded alcohols, phenols
2829.57	2551.5	2823.79	2825.82	2881.65	2881.65	=C-H	Alkanes
--	2350.16	2378.23	2360.87	2376.30	2374.37	C-H Stretching	Alkanes
1889.02	1869.02	--	1869.02	--	1869.02	Unknown	
1635.64	1641.42	1641.42	--	--	--	C=C stretching	Alkene
--	--	--	1543.08	--	1558.48	C=O aromatic stretching	Alkene
--	1095.57	1097.57	--	1093.84	1093.64	C-O	Ester
874.05	974.95	974.05	974.05	--	974.05	C-H bending	Alkane
798.53	798.53	798.53	798.53	798.53	798.53	aromatic carbons	
--	--	--	--	466.77	466.77	S-S	Aryl disulphides

Phytochemical analysis of callus tissue by GC-MS

Phytochemical screening of callus is required to identify the nature of bioactive components in order to find novel therapeutic agents with better efficacy. The spectral peaks in the chromatogram were compared with the spectrum of known compounds stored in the NIST library. The identified compounds, their molecular formulae, molecular weight, retention time (RT), and percentage composition (% area) are given in **Table 3**. A distinct chromatogram of callus tissue extract of *R. serpentina* is shown in **Figure 10**. The structure of individual components is illustrated in **Table 3**. In the present study, GC-MS chromatogram confirmed the presence of 26 different peaks thus, suggesting 26 compounds with their respective RT (**Figure 10**) in the callus extract of *R. serpentina*. The phytocomponents in the callus tissue extract of *R. serpentina* showed a chromatogram with retention time ranging from 3.99 to 18.44. Among the identified 26 phytocomponents from the ethyl acetate extract of *R. serpentina* callus tissue (**Table 3**), the most abundant components were gamma sitosterol (100%), hentriacontane (42.92) followed by phytol, acetate (42.18), resorcinol (35.65%), N-Benzyl-2-phenethylamine (32.15%), cholest-5-en-3-ol, 24-propylidene-, (3.beta.)- (31,95%), pentacosane (31.68%), betulin (31.11%), stigmasterol (28.35%), ethyl iso-allocholate (27.55%), 7-Methyl-Z-tetradecen-1-ol acetate (24.14%) and vitamin E (21.35%). The biological activities of the phytoconstituents are listed in **Table 4**. Based on the spectral data, it was found that the extract of callus of *R. serpentina* contained a large number of bioactive compounds including phytosterols

and fatty acids. Most of these phytoconstituents have been reported with pharmacological activity. The presence of various bioactive compounds justifies the propagation and use of this callus tissue for phytopharmaceutical purposes.

Table 3: Phytoconstituents present in *R. serpentina* as identified using GC-MS.

Peak	Retention Time	Name of the compound (IUPAC Names)	*Molecular formula*	Molecular Weight	Area %
1	3.995433	N-Benzyl-2-phenethylamine	$C_{15}H_{17}N$	211.3	32.15
2	4.167033	Resorcinol	$C_6H_6O_2$	110.1	35.65
3	6.7824	Phytol, acetate	$C_{22}H_{42}O_2$	338.6	42.18
4	6.938933	Phthalic acid, butyl undecyl ester	$C_{23}H_{36}O_4$	376.5	10.76
5	7.061483	Phytol, acetate	$C_{22}H_{42}O_2$	338.6	11.16
6	8.622817	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.4	3.66
7	8.707683	Phytol	$C_{20}H_{40}O$	296.5	17.12
8	10.14835	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366.7	9.58
9	11.66262	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.4	24.14
10	11.94737	Di-n-octyl phthalate	$C_{28}H_{38}O_4$	518.6	22.64
11	12.34337	Ethyl iso-allocholate	$C_{13}H_{14}N_2O_3$	246.3	27.55
12	12.88455	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568.9	5.6
13	13.11273	Pentacosane	$C_{25}H_{52}$	352.7	31.68
14	13.96888	Supraene	$C_3H_2F_6O$	168	16.56
15	14.48182	Hentriacontane	$C_{31}H_{64}$	436.9	42.92
16	15.75653	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	917.2	20.85
17	15.91305	Vitamin E	$C_{29}H_{50}O_2$	430	21.35
18	16.45428	Campesterol	$C_{28}H_{48}O$	400.7	10.04
19	16.65603	Stigmasterol	$C_{29}H_{48}O$	412.7	28.35
20	16.99923	gamma-Sitosterol	$C_{29}H_{50}O$	414.7	100
21	17.11423	Cholest-5-en-3-ol, 24-propylidene-, (3.beta.)-	$C_{30}H_{50}O$	426.7	31.95
22	17.184	Betulin	$C_{30}H_{50}O_2$	442	7.22
23	17.33673	3,9-Epoxypregn-16-en-20-one, 3-methoxy-7,11,18-triacetoxy	$C_{28}H_{38}O_9$	518.6	7.26
24	17.4366833	Betulin	$C_{30}H_{50}O_2$	442	31.11
25	17.7874	3,9-Epoxypregn-16-en-20-one, 3-methoxy-7,11,18-triacetoxy	$C_{28}H_{38}O_9$	518.6	12.2
26	18.43797	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	579.2	7.78

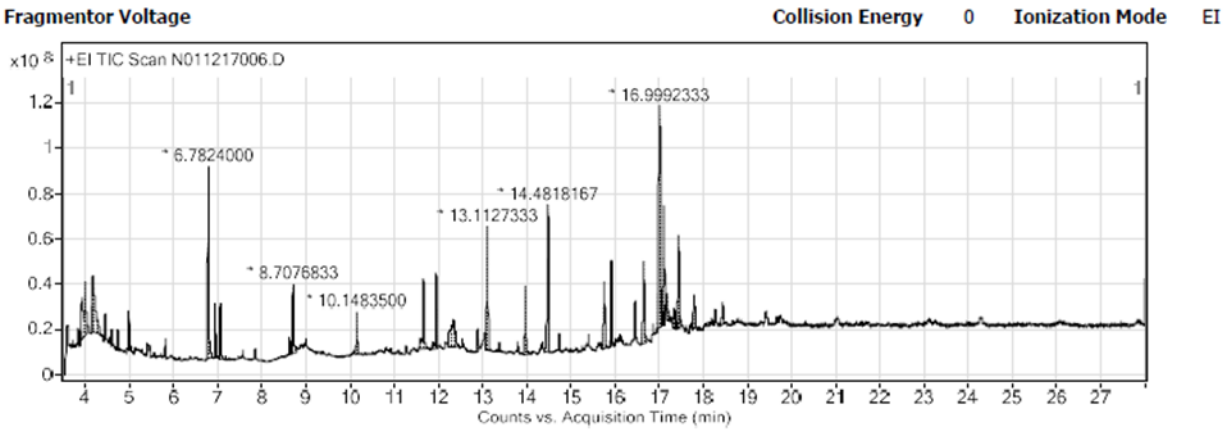

Figure 10: GC chromatogram of ethyl acetate extract of callus tissue of *R. serpentine.*

Table 4: Bioactivity of phytocomponents identified in the ethyl acetate extract of callus tissue of *R. serpentina* by GC MS.

Peak	Name of the compound	Nature of the compound	Biological activities	Reference
1	N-Benzyl-2-phenethylamine	Aromatic amine	5-$HT_{2A/2C}$ Agonist	[33]
2	Resorcinol	Benzene diol	Used in hair dye formulation, goiterogenic, antiseptic and disinfectant used in the treatment of skin disorders and infections such as acne, seborrheic dermatitis, eczema, psoriasis, corns, calluses and warts	[34]
3	Phytol, acetate	Fatty alcohol	Anti-inflammatory, antileishmanial and antitrypanosomal	[35]
4	Phthalic acid, butyl undecyl ester	Ester	Antimicrobial, anti-inflammatory	[35]
5	Phytol, acetate	Fatty alcohol	Anti-inflammatory, antileishmanial and antitrypanosomal	[35]
6	7-Methyl-Z-tetradecen-1-ol acetate	Acetate ester	Anti-inflammatory, hepatoprotective, anticancer	[36,37]
7	Phytol	Diterpene	Antioxidant, anticancer, antimicrobial, antidiuretic anti-inflammatory, immune-stimulatory, antiarthritic, antioxidant and antidiabetic	[38-40]
8	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	Alkane	Antimicrobial, antifungal	[41]
9	7-Methyl-Z-tetradecen-1-ol acetate	Acetate ester	Anti-cancer, anti-inflammatory, hepatoprotective	[36,37]
10	Di-n-octyl phthalate	Ester	Antimicrobial, anti-inflammatory, antioxidant	[42]

11	Ethyl iso-allocholate	Steroid	Antimicrobial, antioxidant anti-inflammatory, antiarthritic, antiasthmatic	[43]
12	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Fatty acid ester	Antibacterial, antifungal, nematicide, pesticide, lubricant, anti-androgenic, antifibrinolytic, hemolytic hypocholesterolemic, anti-alopecic, antioxidant, 5-alpha reductase inhibitor	[44,45]
13	Pentacosane	Aliphatic hydrocarbon	antibacterial	[46]
14	Supraene	fluorinated ether	anesthetic and muscle relaxant	[47]
15	Hentriacontane	Long chain alkane hydrocarbon	Anti-inflammatory, diuretic, anti-tubercular	[48]
16	Tetrapentacontane, 1,54-dibromo-	Hydrocarbon	Used in fermentation, gives aroma	[49]
17	Vitamin E	Steroid	Antioxidant, antiaging, hypocholesterolemic, antialzheimer, analgesic, antidermatitic, antidiabetic, antitumor, cancer preventive, anti-leukemic, antibronchitic, immunostimulant, anti-inflammatory, antiulcerogenic, vasodilator, anticoronary antispasmodic,	[50-52]
18	Campesterol	Steroid	Anti-inflammatory, anti-tumor, cancer preventive, antimicrobial, diuretic. antioxidant, antiarthritic anti-inflammatory, anti-asthma, hepatoprotective, hypocholesterolemic	[35]
19	Stigmasterol	Steroid	Antioxidant, antidiabetic, antiviral, antimicrobial, hypoglycemic, diuretic. anticancer, antiarthritic, antiasthma, thyroid inhibitory, cancer preventive, antihepatotoxic, anti-inflammatory, hypocholesterolemic, Precursor of progesterone	[53-57]
20	gamma-sitosterol	Steroid	Antidiabetic, angiogenic, antimicrobial, antiviral hepatoprotectant, anti-cancer, anti-diarrheal, antiinflammatory	[58]
21	Cholest-5-en-3-ol, 24-propylidene-, (3.beta.)-	Steroid	No activity reported.	

22	Betulin	Pentacyclic lupane type triterpenoid	anticancer, apoptotic,anti-HIV, antibacterial, antimalarial, anti-inflammatory, anthelmintic, anti-HSV-1. antinociceptive	[59-61]
23	3,9-Epoxypregn-16-en-20-one, 3-methoxy-7,11,18-triacetoxy	Steroid	No activity reported.	
24	Betulin	Pentacyclic lupane type triterpenoid	anticancer, apoptotic, anti-HIV, antibacterial, antimalarial, anti-inflammatory, anthelmintic, anti-HSV-1. antinociceptive	[59-61]
25	3,9-Epoxypregn-16-en-20-one, 3-methoxy-7,11,18-triacetoxy	Steroid	No activity reported.	
26	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	Volatile organic compound	Antimicrobial	[62]

Conclusions

Taken together, the present study provided a rapid protocol for callus initiation and growth from leaf explants of *R. serpentina*. For the first time, α-amylase was found in the callus extract by SDS-PAGE and confirmed by Western blot. The fourth band of TLC from the ethyl acetate extract as well as silver and gold nanoparticles synthesized using this extract revealed pronounced antimicrobial and antioxidant activities. GC-MS analysis revealed 26 compounds, which included mainly the phytosterols and fatty acid esters. The presence of these compounds in the callus tissue of *R. serpentina indicates* that they are promising candidates for therapeutic use.

Acknowledgement

We thank the Vice President, Chancellor and Vice Chancellor, Kalasalingam University, Krishnankoil, Viruthunagar District, Srivilliputhur-626126 Tamil Nadu, India for their constant encouragement to carry out this work.

Conflict of interest

The authors declare no conflict of interest.

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Citation: *Kishore RP, Vikram V, Sundaram LR, et al. Evaluation of Antioxidant and Antibacterial Potential and α-Amylase Expression in the Leaf Callus Tissue of Rauvolfia serpentina (Linn.) Benth. Ex Kurz. Int J Pharm Pharmacol 2020; 4: 144. doi:10.31531/2581-3080.1000144*

This manuscript was peer-reviewed
Mode of Review: Single-blinded
Academic Editor: Dr. Syed Sarim Imam