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(RESEARCH ARTICLE)

Evaluation of *α-amylase* expression and analysis of phytochemical in the leaf callus tissue of *Rauvolfia serpentina* (Linn.) Benth. Ex Kurz exposed to Cyclodextrin

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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 the regeneration of leaf explants from *R. serpentina* and to study the α-amylase expression and phytochemical profiling by gas chromatography and mass spectrometry (GC-MS). Further antibacterial activity was studied using silver, gold, and copper nanoparticles. 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 with underexposed to Cyclodextrin. Explants growing on MS 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 a maximum concentration of protein on the 75th day. Synthesis of a-amylase enzyme was expressed 40 days old culture and were confirmatic by western, further 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-negative and positive bacteria and potent antioxidant activity. 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.

Keywords: MS medium; α-amylase; Benzyl amino purine; Thin layer chromatography; Gas chromatography and mass spectrometry

1. Introduction

Rauvolfia serpentina (Linn.) Benth. Ex Kurz, popularly known as Sarpagandha, Indian snake root or devil pepper belongs to the family Apocynaceae. More than 100 species are included in the *Rauwolfia* genus, and they are native to tropical and subtropical regions of the world, including Europe, Africa, Asia, Australia and the Central and South America. *R. serpentina* is native to the moist, deciduous forests of Southeast Asia, including India, Burma, Bangladesh, Srilanka and Malaysia. *R. serpentine* is an ever green glaborous shrub which grows up to 60 m of height.

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R. serpentina is widely used in Ayurveda, Unani, folk medicine and allopathic system. *R. serpentina* has been used since pre-Vedic period as an antidote against the bites of poisonous snakes and insect stings (Anonymous, 2006). In folk and tribal medicine, the root extracts are used as laxative, uterine stimulant, diuretic, antihelmintic, antidote, expectorant and febrifuge (Ghani, 1998; Fabricant and Farnsworth, 2001; Rai, 2004). 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 (Ghani, 1998; Rai, 2004; Dey and De, 2010; Azmi *et al*., 2012).

R. serpentine is a rich source of different varieties of chemical constituents. The root of this plant contains several alkaloids; which include ajmalicine, reserpine, serpentinine, ajmaline, ajmalimine, deserpidine, indobidine, reserpiline, rescinnamine, rescinnamidine, serpentine, and yohimbine (Anonymous, 2001; Day and De, 2011). Among the alkaloids, reserpinehas attracted worldwide attention for drug development. It is also useful in treating sedative insomnia, psychological disorders, excitement, epilepsy, traumas, anxiety, schizophrenia, insanity and in reducing blood pressure (Rai, 2004; Itoh et al., 2005; Dey and De, 2010; Azmi *et al*., 2013). Reserpine exerts antihypertensive property by depleting the catecholamine (Gawade and Fegade, 2012; Singh *et al*., 2015). Rescinnamine has the same activity like 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 (Gawade and Fegade, 2012; Singh *et al*., 2017). 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 (Singh *et al*., 2017). High concentration of phenols of *R. serpentina* revealed significant antidiabetic, hypolipidemic and antimicrobial properties. Flavonoids of *R. serpentine* help in preventing the oxidative cell damage and having anticancer, anti-inflammatory, and antioxidant properties (Harisaranraj *et al*., 2009; Deshmukhey *et al*., 2012; Kumari *et al*., 2013). The presence of saponins is responsible for the hemolytic activity and cholesterol binding property (Kokate, 2012).

Ethnopharmacological studies have shown the antioxidant activity with respect to superoxide anion scavenging activity, reducing power and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity by the methanolic extract of leaves of *R. serpentina* (Nair *et al*., 2012). Methanolic extract of *R. serpentina* rhizome also exhibited antioxidant activity as evident by the free radical scavenging activity and the increased level of glutathione peroxide, glutathione-S-transferase, glutathione reductase, superoxide dismutase, catalase, glutathione and decreased level of lipid peroxidation in CCl_4-induced hepatotoxicity rat model (Gupta *et al*., 2015). Ethanolic extract of root was shown to possess antibacterial activity against *Staphylococcus, Bacillus subtilis* (Gram-positive) and *Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Salmonella typhimurium* (Gram-negative bacteria) (Harisaranraj *et al.,* 2009; Negi et al., 2014; Murthy and Narayanappa, 2015). Ethanolic extract of *R. serpentina* whole plant showed antivenom activity by neutralizing the toxic effect of *Najanaja* venom (Rajashree *et al*., 2013). Aqueous ethanolic extract of the root of *R. serpentina* manifested hepatoprotective activity by protecting the liver from paracetamol-induced liver toxicity in rats (Gupta *et al.,* 2010). 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 (Gupta *et al.,* 2010). Azmi *et al*. (2012; 2015) 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*. (2012) evaluated the antidiarrheal property of methanolic extract of leaves of *R. serpentina* in castor oil-induced diarrhea in mice.

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 Fourier Transform Infrared (FTIR) Spectroscopy and screened for antimicrobial and antioxidant activities.

2. Material and methods

2.1. Source of explants of *R. serpentina*

Leaf segments of *R. serpentina*. obtained from healthy mother plant (1-2 months old) (Fig.1) growing in the Kalasalingam Academy of Research and Education, Krishnankoil, Tamil Nadu, India, served as explants (Figs 1a-f).

2.2. 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 surface-sterilized with mercuric chloride (0.1 %) for 5 min and then rinsed with distilled water five-six times.

2.3. Preparation of culture media and culture conditions

Tissue culture medium was prepared according to the method of Murashige and Skoog (1962). 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 in an autoclave at 121°C under 15 psi for 15 min.

Under laminar flow cabinet, disinfected leaves were aseptically excised and placed on the media in different orientations. The cultures were maintained at 27ºC with 16 h light and 8 h dark 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.

2.4. 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}).

2.5. 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 (1976) using BSA as a standard.

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.

2.6. 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 with the horseradish peroxidase–conjugated secondary antibody for 2 h at room temperature. 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**.**

2.7. 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 desicator until use.

2.8. 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 with constant stirring at room temperature. The mixtures were heated at 60°C and then cooled to room temperature and kept in dark for 24 h. The color change of the mixture 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).

2.9. 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 hexane and ethyl acetate (8:2) as the mobile phase. 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.

2.10. Antibacterial activity

The antibacterial activity was checked by TLC bioautographic method using 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 subcultured 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, mixed with 5 ml of absolute ethanol, allowed to stand for 10 min and then filtered with Whatman No. 1 filter paper and 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 inoculam (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 *E.coli* (Gram negative) and *S. aureus* (Gram positive)bacteria.

2.11. 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 (Prieto *et al*., 1999).

Total antioxidant capacity was assessed by phosphomolybdenum method. The assay is based on the reduction of molybdenum (VI) to green phosphate/molybdenum (V) complex at acidic pH by the sample analyte.

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

2.12. 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 spectral 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 was then encapsulated using KBr (100 mg) pellets in order to prepare translucent sample discs by applying pressure for FTIR analysis.

2.13. 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 6˚C/min. Helium was used as the carrier gas 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.

2.14. Identification of components

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

2.15. 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.

3. Results and discussion

3.1. 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 (Pandey *et al*., 2010; Shetty *et al*., 2014; Chaudhury *et al*., 2016). 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 (Pand and Joshi, 2008). MS media without plant growth regulators failed to induce callus (Salma *et al.,* 2008). In the present study, a combination of BAP and NAA in the medium significantly influenced the induction of callus (Figs 1a-f). This is consistent with the report of Pandey *et al*. (2010) 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 (Pandey *et al*., 2010). Rashmi and Trivedi (2016) 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 considered optimal for the induction and growth of callus (Figs 1a-f).

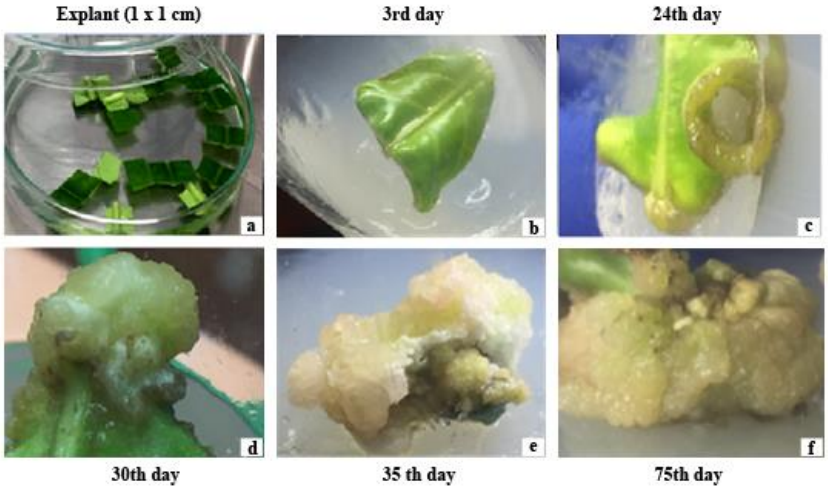


Figure 1 Different stages of callus development in MS media supplemented with BAP (2.0 mg/L) and NAA (0.1 mg/L)

3.2. 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 (Fig.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 (Fig. 2b).

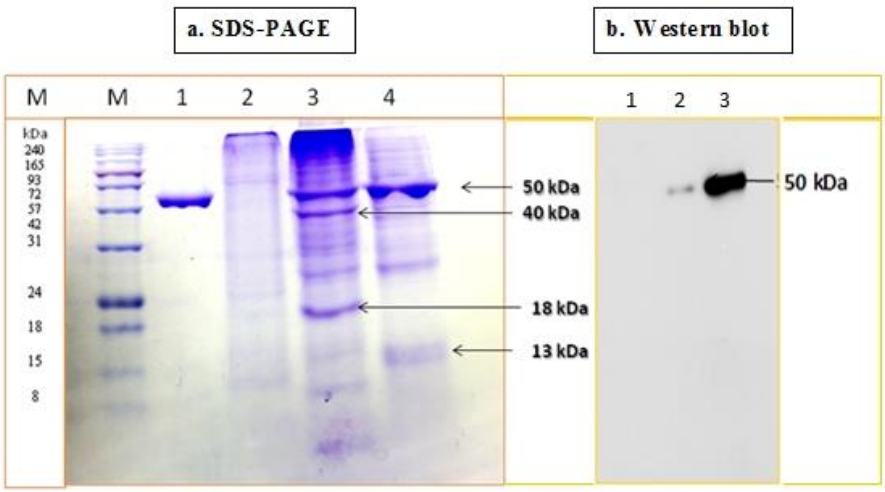


Figure 2 Separation of protens by SDS-PAGE and amylase expression by Western blot [Fig. 2a Separation of protein samples run on 12% SDS-PAGE (M-Marker, 1-α-amylase alone, 2-crude leaf protein, 3-crude leaf callus protein, and 4-crude root protein) 2b. Western blot assay (1-leaf protein, 2-leaf callus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

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

S.No	Time points	Protein concentration (mg)	
		Auxins and cytokinins	
		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 ± 0.03a	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 $p < 0.005$

3.3. Characterisation of silver and gold nanoparticles

SEM analysis revealed that silver nanoparticles synthesized through green chemistry are well dispersed and spherical in shape (Figs 4a and b). Fig. 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 (Fig. 5a). EDX analysis revealed the presence of silver and gold elements, confirming the successful synthesis of silver and gold nanoparticles (Figs 4c and 5c).

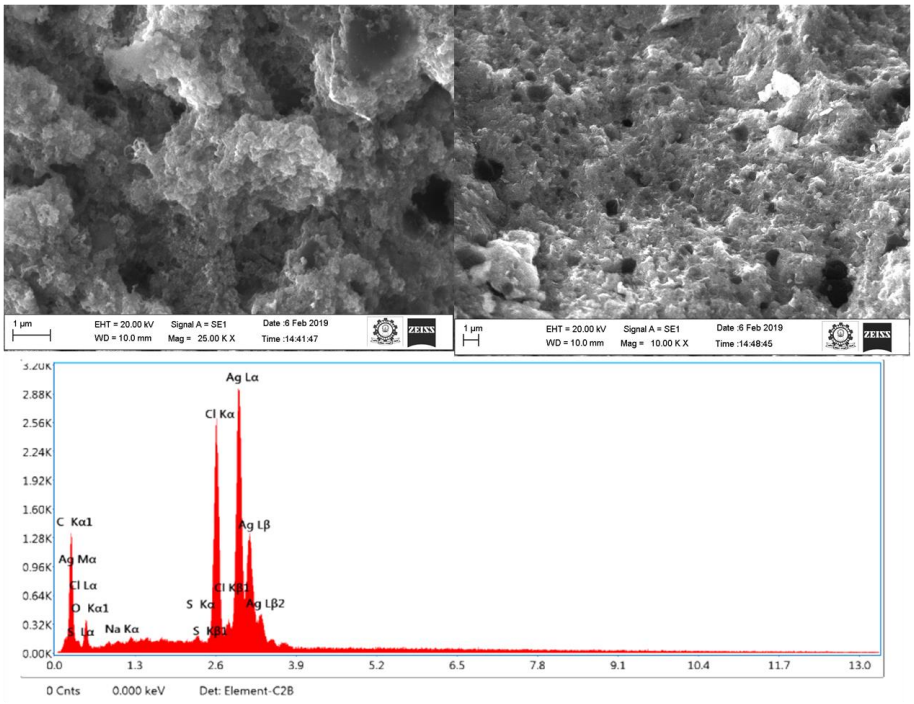


Figure 4 SEM images with EDX analysis of silver nanoparticles synthesized using ethyl acetate extract of callus of R. serpentine [Scanning Electron microscopy images (a and b) represents silver nanoparticles synthesised using ethyl acetate extract of callus of *R. serpentina*. Fig. 4c represents the Energy-Dispersive X-ray microanalysis of silver nanoparticles].

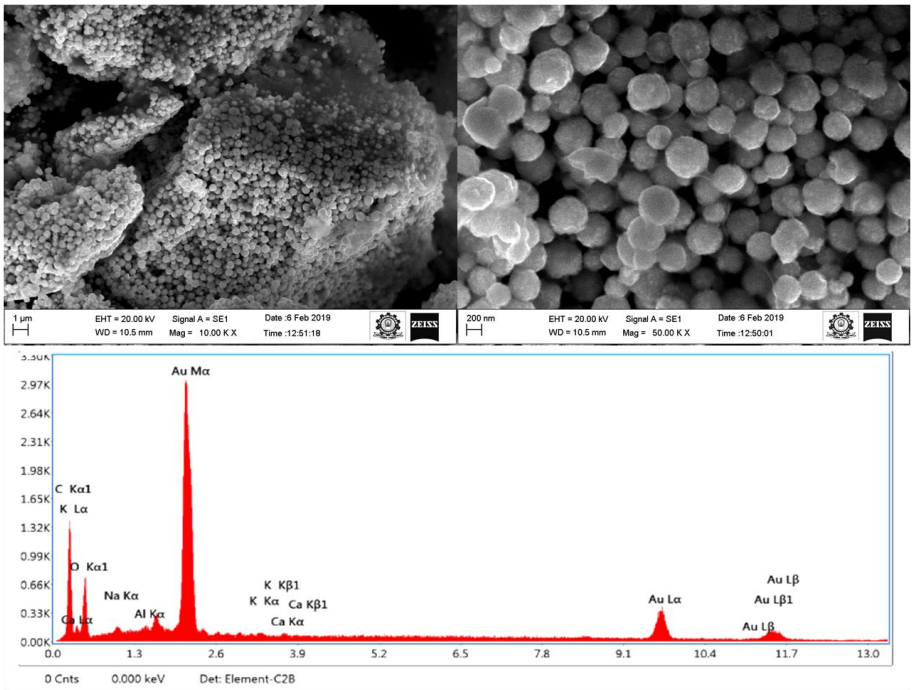


Figure 5 SEM images with EDX analysis of gold nanoparticles synthesized using ethyl acetate extract of callus of *R. serpentine* [Fig.5 Characterisation of gold nanoparticles. Scanning Electron microscopy images (a and b) represents gold nanoparticles synthesised using ethyl acetate extract of callus of *R. serpentina.* Fig. 5c represents the Energy-Dispersive X-ray microanalysis of gold nanoparticles.]

3.4. 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.

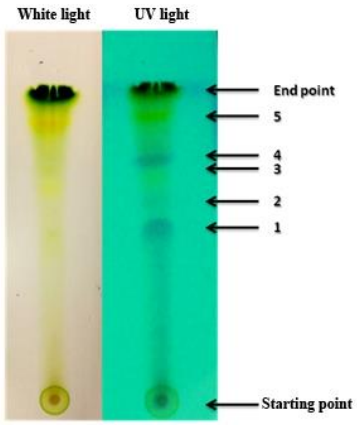


Figure 3 TLC chromatogram showing bands under white light and UV light

In the present study, TLC chromatogram revealed five bands (Fig.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 (Fig.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. serpentine* (Negi *et al*., 2014; . Murthy and Narayanappa, 2015).

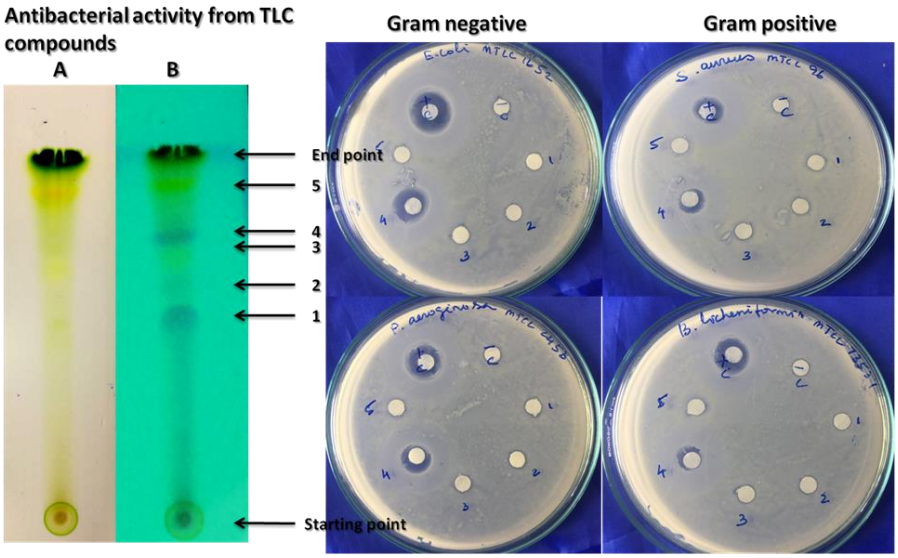


Figure 6 Antibacterial activity by TLC bioautographic method [Fig.6 Antibacterial activity by TLC bioautographic method; TLC chromatogram showing five bands. Antibacterial activity was checked using the concentrates of five TLC bands against Gram negative (a) *E. coli* (MTCC 1652), (b) *P. aeroginasa*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

The antibacterial effects of three nanoparticles (gold, copper and silver) using ethyl acetate extract of callus tested against Gram negative (*E. coli*) and Gram positive bacteria (*S. aureus*) are presented in Fig. 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 (2015), who have shown maximum antibacterial activity of leaf or root extracts of *R. sepentina* for Gram negative bacteria than Gram positive bacteria.

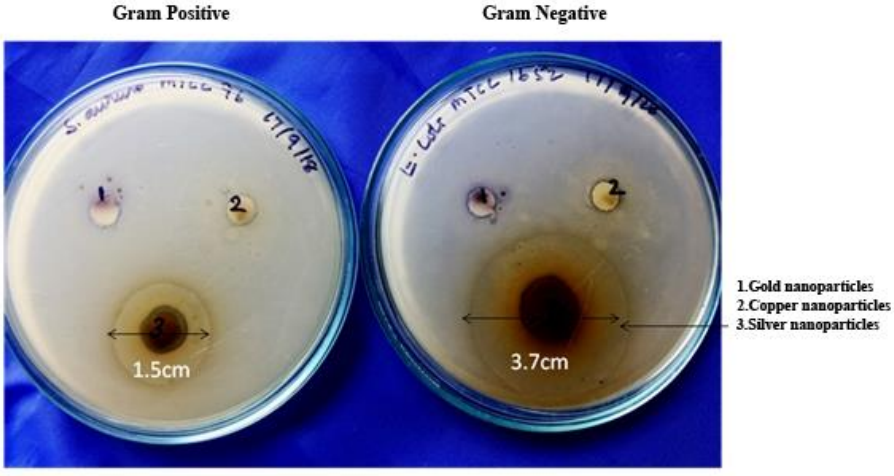


Figure 7 Antibacterial activity of gold, copper and silver nanoparticlesusing ethyl acetate extract of callus tissue of *R. serpentina* [Fig.7 Antibacterial activity of gold, copper and silver nanoparticles using ethyl acetate extract of callus tissue of *R. serpentina.* Antibacterial activity shown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

3.5. 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 (Fig. 8).

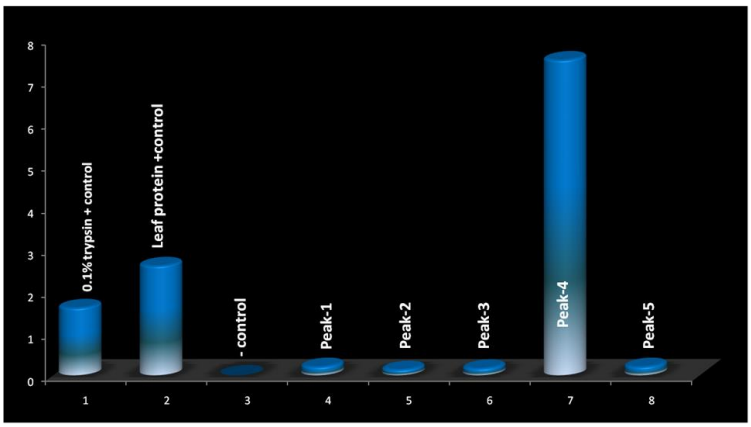


Figure 8 Antioxidant activity by TLC bioautographic method using ethyl acetate extract of callus tissue of *R. serpentina* [Values are expressed as mean ± SEM; a – Compared to Control; b – Compared to ascorbic acid; Significant at $p < 0.005$]

3.6. 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 Fig.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 (Fig. 9a and 9b).

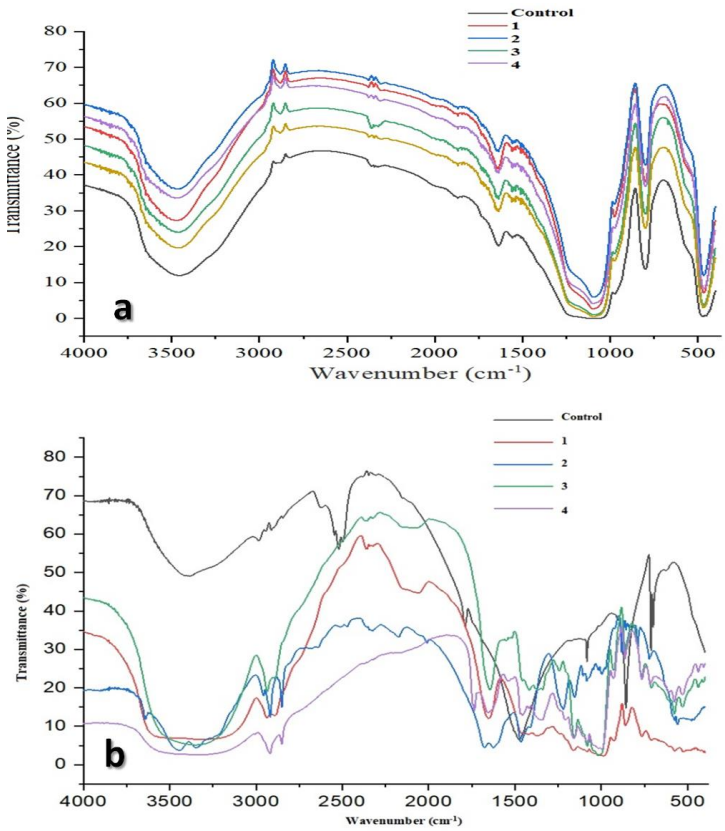


Figure 9 Characterization of five TLC bands by FTIR (a) without underexposed to cyclodextrin; and (b) with underexposed to cyclodextrin

The samples were analyzed in the spectral region of 500 to 4000 cm^{-1}. All the five bands of TLC exhibited a characteristic absorption maxima 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).

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. serpentina*

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 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 retention time (RT), molecular weight, molecular formulae, and percentage composition (% area) are given in Table 3. A distinct chromatogram of callus tissue extract of *R. serpentina* is shown in Figure 10.

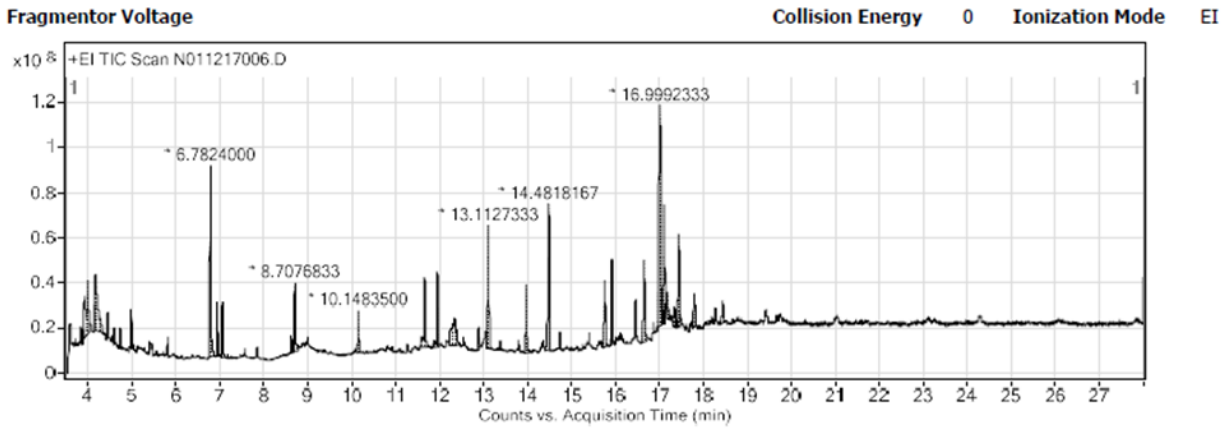
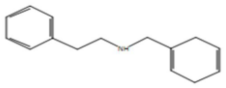
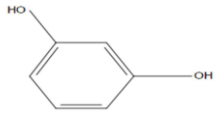
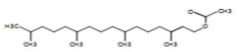
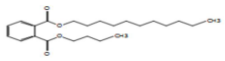
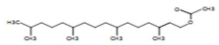
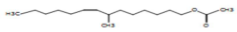
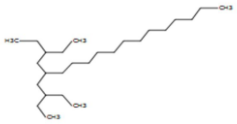
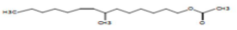
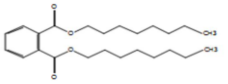
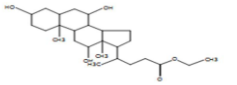


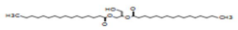
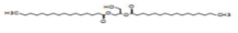
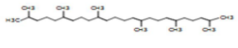
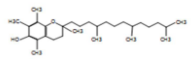
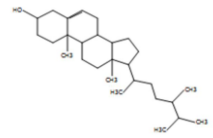
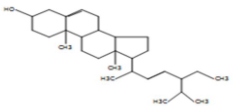
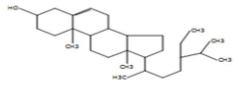
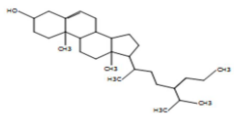
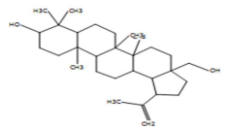
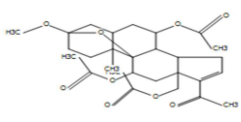
Figure 10 GC chromatogram of ethyl acetate extract of callus tissue of *R. serpentina*

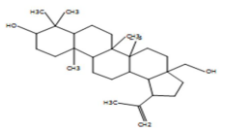
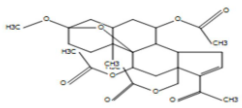
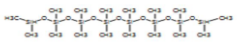
The structure of individual components is illustrated in (Table 3). In the present study, GC-MS chromatogram shows the presence of 26 different peaks which confirm the presence of 26 compounds with their respective RT (Fig. 10) in the callus extract of *R. serpentina.* The phytocomponents in the callus 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%).

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

Peak	Retention time	Name of the compound (IUPAC Names)	Molecular formula	Molecular weight	Area %	Structure of the compound
1	3.9954333	N-Benzyl-2-phenethylamine	$C_{15}H_{17}N$	211.3	32.15	
2	4.1670333	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	6.9389333	Phthalic acid, butyl undecyl ester	$C_{23}H_{36}O_4$	376.5	10.76	
5	7.0614833	Phytol, acetate	$C_{22}H_{42}O_2$	338.6	11.16	
6	8.6228167	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.4	3.66	
7	8.7076833	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	11.6626167	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268.4	24.14	
10	11.9473667	Di-n-octyl phthalate	$C_{28}H_{38}O_4$	518.6	22.64	
11	12.3433667	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	13.1127333	Pentacosane	$C_{25}H_{52}$	352.7	31.68	
14	13.9688833	Supraene	$C_3H_2F_6O$	168.0	16.56	
15	14.4818167	Hentriacontane	$C_{31}H_{64}$	436.9	42.92	
16	15.7565333	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	16.4542833	Campesterol	$C_{28}H_{48}O$	400.7	10.04	
19	16.6560333	Stigmasterol	$C_{29}H_{48}O$	412.7	28.35	
20	16.9992333	gamma.-Sitosterol	$C_{29}H_{50}O$	414.7	100	
21	17.1142333	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	17.3367333	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	18.4379667	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	

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. serpentine* contained a large number of bioactive compounds including phytosterols and fatty acids. Most of these phytoconstituents are reported to possess pharmacological potential. The presence of various bioactive compounds justifies the propagation and use of this callus tissue for phytopharmaceutical purposes.

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	Hansen et al., 2014
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	WHO 2006
3	Phytol, acetate	Fatty alcohol	Anti-inflammatory, antileishmanial and antitrypanosomal	Al-Marzoqi et al., 2016
4	Phthalic acid, butyl undecyl ester	Ester	Antimicrobial, anti-inflammatory	
5	Phytol, acetate	Fatty alcohol	Anti-inflammatory, antileishmanial and antitrypanosomal	Al-Marzoqi et al., 2016
6	7-Methyl-Z-tetradecen-1-ol acetate	Acetate ester	Anti-inflammatory, hepatoprotective, anticancer	Hameed et al., 2015
7	Phytol	Diterpene	Antioxidant, anticancer, antimicrobial, antidiuretic anti-inflammatory, immune-stimulatory, antiarthritic, antioxidant and antidiabetic	Ogunlesi et al., 2009 Alagammal et al., 2012 Rajesh et al., 2017
8	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	Alkane	Antimicrobial, antifungal	Rao et al., 2015
9	7-Methyl-Z-tetradecen-1-ol acetate	Acetate ester	Anti cancer, anti-inflammatory, hepatoprotective	Hameed et al., 2015
10	Di-n-octyl phthalate	Ester	Antimicrobial, anti-inflammatory, antioxidant	Ibraheam et al., 2017
11	Ethyl iso-allocholate	Steroid	Antimicrobial, antioxidant anti-inflammatory, antiarthritic, antiasthmatic	Sheela and Uthayakumar, (2013)
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	Venkataraman et al., 2012; Aneesh et al., 2013 Markkas et al., 2015

13	Pentacosane	Aliphatic hydrocarbon	antibacterial	Mihailović et al., 2011
14	Supraene	fluorinated ether	anesthetic and muscle relaxant	Magni et al., 2009
15	Hentriacontane	Long chain alkane hydrocarbon	Anti-inflammatory, diuretic, anti-tubercular	Kim et al., 2011
16	Tetrapentacontane, 1,54-dibromo-	Hydrocarbon	Used in fermentation, gives aroma	Zhu et al., 2012
17	Vitamin E	Steroid	Antioxidant, antiaging, hypocholesterolemic antialzheimeran, analgesic, antidermatitic, antidiabetic, antitumor, cancer preventive, anti leukemic, antibronchitic, immunostimulant, anti-inflammatory, antiulcerogenic, vasodilator, anticoronary antispasmodic,	Kayden and Wisniewski, 2000; Traber and Atkinson, 2007; Rizvi et al., 2014
18	Campesterol	Steroid	Anti-inflammatory, anti-tumor, cancer preventive, antimicrobial, diuretic. antioxidant, antiarthritic anti-inflammatory, anti asthma, hepatoprotective, hypocholesterolemic	Al-Marzoqi et al., 2016
19	Stigmasterol	Steroid	Antioxidant, antidiabetic, antiviral, antimicrobial, hypoglycemic, diuretic. anticancer, antiarthritic, antiasthma, thyroid inhibitory, cancer preventive, antihepatotoxic, anti-inflammatory, hypocholesterolemic, Precursor of progesterone	Panda et al., 2009; Gabay et al., 2010; Ekade and Manik, 2014; Dandekar et al., 2015; Subbaiyan et al., 2015
20	gamma-sitosterol	Steroid	Antidiabetic, angiogenic, antimicrobial, antiviral hepatoprotectant, anti cancer, anti-diarrheal, anti-inflammatory	Karthikeyan et al., 2017
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	Cichewicz and Kouzi (2004); Alakurtti et al., 2006; Moghaddam et al., 2012
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	Cichewicz and Kouzi (2004); Alakurtti et al., 2006; Moghaddam et al., 2012
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	Kumaradevan et al., 2015

4. Conclusion

Taken together, the present study provided a rapid protocol for callus initiation and growth from leaf explants of *R. serpentina* exposed to cyclodextrin in MS medium. 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 and food supplementation of nutraceuticals.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors do not have any conflicts of interest.

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