BIOSYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING CATHARANTHUS ROSEUS AQUEOUS EXTRACT

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Abstract - The biosynthesis of nanoparticles has been proposed as a cost-effective and environmental friendly alternative to chemical and physical methods. The present study was aimed to synthesise and characterise Catharanthus roseus (C. roseus) silver nanoparticles (AgNPs). The extract were prepared by soaking the dried leaves of C. roseus in 40 ˚C water bath for 24 h or boiled for 5 min. The extracts at concentrations of 10% or 20% were mixed with various concentrations of $AgNO_3$ solutions (1, 3, 5 and 10 mM). The characterisation of C. roseus-AgNPs was performed by ultraviolet-visible (UV-Vis) spectra analysis, transmission electron microscope (TEM), X-ray diffraction (XRD), zeta potential analysis, fourier transform infrared spectroscopy (FTIR), and Liquid Chromatography/Time-of-Flight ion trap Mass Spectrometry (LC/TOF-MS). The colour changes indicate the presence of AgNPs in the solution, resulting from the reduction of Ag^+ ions into AgNPs. UV-Vis spectra analysis indicates that AgNPs at concentration of 5 mM $AgNO_3$ and 10% extract has produced the highest surface plasmon resonance peak at approximately 450 nm of wavelength. The shape and size determined by TEM indicates that the AgNPs sizes were in the range of 20 to 50 nm (average size of 31 nm with spherical shape). The XRD analysis confirmed the face-centered cubic structure of AgNPs. Zeta potential analysis exhibited the value at -16.8 mV, suggesting high stability of AgNPs due to the capping of biomolecules present in the C. roseus aqueous extract. FTIR spectrum of C. roseus aqueous extract showed the absorption band at 3210.83 cm^{-1} (C-H stretch), 2934.11 (C-H bond), 1578.15 (N=O stretch), 1388.76 & 1314.89 (N=O bend), 1119.29 (C-O bond) and 729.94 (C-Cl bond). While, the FTIR spectrum of C. roseus-Agnps showed the absorption band at 2925.01 & 2924.97 (C-H bond), 1622.93 (C-C=C symmetric stretch), 1383.19 & 1384.13 (N-O bend), 1037.92/1038.76/1238.3/1117.2 (C-O bond), 3169.4 (O-H bond), 774.59 & 691.53 (C-Cl bond). LC/TOF-MS confirmed the presence of indole alkaloids which were vindolinine, tabersonine, catharanthine, serpentine, catharosine, vincristine, catharine, ajmalicine, vinleurosine, and vindolicine. The present findings have shown that the AgNPs have been successfully synthesised and contain anticancer compounds that responsible for its synthesis.

Index Terms - Catharanthus roseus, characterisation, LC/TOF ion trap MS, silver nanoparticles

I. INTRODUCTION

Recently, the development of green synthesis of nanoparticles is evolving into an important branch of nanotechnology [1]. The green syntheses are not only a good way to fabricate nanostructure materials, also reducing the use of substances hazardous to human health and the environment [2],[3]. The research on biosynthesised nanomaterials and their characterisation is gaining more and more attention due to their wide applicability, especially in biomedical fields (e.g., in the diagnosis and treatment of human cancers) [4]. The special characteristics of nanomaterials and their biologic effects suggest that nanoparticles may become potential alternative treatments of disease [5].Ultrafine particles of metallic silver at the nanometer (nm) scale were found to exhibited distinctive morphologies and characteristics [5]. Traditional methods of synthesising nanoparticles include radiation, chemical or photochemical methods, electrochemical techniques, and Languir-Blodgett approaches [2]. However, these methods are extremely expensive and time consuming and can be dangerous to human health and the environment because of the application of hazardous substances [6],[7]. Therefore, there is a growing need to develop cost-effective and environmentally friendly approaches for rapid synthesis of nanoparticles.

Biological approaches to the synthesis of metal nanoparticles using microorganisms and plant extracts have been suggested as valuable alternatives to chemical synthesis and physical methods [8],[9]. Reference [2] have described the use of natural materials such as plants, bacteria, fungi, yeast, and honey for synthesising gold and silver nanoparticles. For example, studied the applicability of bacteria Bacillus cereus [10] and on fungal species Trichoderma asperellum [11] for synthesising nanoparticles.

The rate of synthesis of nanoparticles by plant extracts is higher than that of chemical methods and green synthesis by microorganisms [12]. In addition, the use of plant materials for the synthesis of nanoparticles does not require elaborate processes such as intracellular synthesis and multiple purification steps or the maintenance of microbial cell cultures [13]. Moreover, the use of plants for synthesis of nanoparticles is rapid, low cost, eco-friendly, and a

single-step process [14]. Many previous studies reported the biosynthesis of silver nanoparticles (AgNPs) using extracts of leaves of various plants, including Pterocarpus santalinus [4], Moringa oleifera [6], Duranta repens [12], Oleo europaea [15], Loquat leaf extract [16], Annona squamosa [17], Rhinacanthus nasutus [18] and Catharanthus roseus [19]. Catharanthus roseus is an erect procumbent herb or undershrub containing latex that grows up to 1 m tall in subtropical areas [13]. This perennial herb is grown commercially for medicinal uses in India, Australia, Africa, and Southern Europe. It contains alkaloids, mainly of the indole type. The root extract (alkaloid alstonine) is used to reduce hypertension, and reserpine, ajamalicine, and serpentine are alkaloids with antiplasmodic and hypotensive properties [1]. C. roseus also has antibacterial, anti-inflammatory, antidiuretic, cytotoxic, antifertility, hyperglycemic, antifungal, anti-malarial, and antivirus effects [19]. The alkaloids vinblastine and vincristine in C. roseus has been used as anti-cancer drugs in the treatment of different types of cancers, such as lymphomas, Hodgkin's lymphoma, breast cancer, acute lymphocytic leukemia, soft tissue sarcomas, multiple myeloma, and neuroblastoma [2]. This study is aimed to evaluate the ability of extracts made from the dried leaves of C. roseus to synthesize AgNPs and characterized the synthesized AgNPs.

II. MATERIALS AND METHODS

A. Preparation of C. roseus Aqueous Extract

The plant C. roseus was collected from Teluk Air Tawar, Butterworth, Penang. The collected plants were sent to Herbarium Unit, School of Biological Sciences Universiti Sains Malaysia (USM) for plant identification. Two methods will be performed to prepare C. roseus aqueous extract. The fresh leaves will be washed and let dried in 40 °C oven before being ground into powder form. Fifty g of dried leaves powder will be dissolved in 1000 mL distilled water before being proceeded with each method. Method 1 will be performed according to [20], where the mixture will be incubated in water bath shaker at 40 °C for 24 h. Meanwhile Method 2 will be performed according to [4], with slight modification of boiling temperature, where the mixture will be boiled at 100 °C on a hot plate for 5 min. Following the incubation times, either 24 h or 5min, the extracts will be centrifuged at 2000 rpm for 15 min and filtered using Whatman filter paper No 1 (Sartourie, Germany) to remove the debris before being freeze dried by using freeze dryer.

B. Synthesis of Silver Nanoparticles (AgNPs)

Four concentrations of C. roseus-AgNPs solutions will be prepared as shown in Table 1. The different concentrations of C. roseus aqueous extracts and AgNO3 solutions will be prepared by adding in distilled water. Five ml C. roseus aqueous extract (w/v) will be dissolved with 45 mL AgNO3 solution (w/v) in a Scott Duran bottle and left at room temperature for 24 h. A brown-yellow solution will be formed indicating the presence of AgNPs. The solution will then be centrifuged at 6000 rpm for 15 min. The supernatant will be discarded and washed twice with distilled water. The pellet will be dried in oven at 40 ˚C for 48 h.

Concentration of *C. roseus* aqueous extract (w/v)	Concentration of $AgNO_3$ (w/v)
10%	1 mM
10%	3 mM
20%	5 mM
20%	10 mM

Table 1: Concentrations of C. roseus aqueous extracts (w/v) and AgNO3 solutions (w/v)

C. Ultraviolet-Visible (UV-Vis) Spectra Analysis

Four concentrations of C. roseus-AgNPs solutions were characterized by using UV-Vis spectroscopy UV-1650 (Shimadzu, Japan) for 0 h, 2 h, 4 h, 22 h, 24 h and 48 h. The bio-reduction of the silver ions was monitored by measuring the absorbance of 1 ml aliquots of the reaction mixture in a wavelength range between 400-600 nm with 1 nm resolution.

D. Transmission Electron Microscope (TEM)

Size and shape of synthesized C. roseus-AgNPs was observed by using TEM CM12 (Phillips, USA) in School of Biological Sciences, USM. The C. roseus-AgNPs was transferred into a new vial by using spatula. A suspension was made by adding 95% alcohol followed by 15 min ultrasonication by using ultrasonic water bath Elmasonic S 80H (Elma, Germany). A drop of suspension was loaded onto a carbon coated grid and let evaporated before viewing.

E. X-Ray Diffraction (XRD) Analysis

One gram of synthesized C. roseus-AgNPs was sent to School of Materials and Mineral Resources Engineering, USM for phase identification of crystalline material and unit cell dimension using XRD D8 (Bruker, USA). A thin layer of vacuum grease was applied on a zero-reflective fused silica followed by the loading of C. roseus-AgNPs. The fused silica was turned upside down to remove excess powder. The process was repeated until a thin layer of powder has adhered to the fused silica before being loaded into the system, operated at a voltage of 30 kV and a current of 30 mA with CuKα radiation in a θ-2θ configuration.

F. Zeta Potential Analysis

The synthesized C. roseus-AgNPs was sent to School of Chemical Engineering, USM to observe the

colloidal stability in dispersion using Zetasizer Nano ZS (Malvern, UK). Stock solution was prepared with a concentration of 30 mg/ml in 0.001 M KNO_3. Three solutions of 0.001 M KNO_3 at pH 3, 6, and 9 was prepared by adjusting the pH with HNO_3 and 0.01 M KOH. Suspensions with a concentration of 1.5 mg/ml was prepared by mixing 1.0 ml of the stock suspension to 20 ml of pH 3, 6 or 9 KNO_3 solutions. These suspensions wereplaced in ultrasonic bath for 5 min. The suspension was then transferred into the measurement cell. The measurements were performed twice per sample at each pH (3, 6 and 9).

G. Fourier Transform Infrared (FTIR) Spectroscopy

The synthesized C. roseus-AgNPs was sent to School of Chemical Sciences, USM for FTIR analysis. FTIR was used to identify the possible functional groups responsible for the reduction of Ag ion and capping of the bio-reduced silver nanoparticles. FTIR analysis of the dried C. roseus aqueous extract and extractmediated synthesis AgNPs powders were carried out using FT-IR Spotlight 200 (Perkin Elmer, USA).

H. Liquid Chromatography/ Time-of-Flight Ion Trap Mass Spectrometry (LC/TOF-MS)

Analysis were performed on Finnigan LTQ ion trap mass spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA) equipped with binary pump, a UV detector, an autosampler, and a column thermostat. Chromatographic separations were carried out on a ZORBAX Eclipse XDB-C_{18} Analytical (5.0 µm, 150 × 4.6 mm, Agilent USA). Elution was perform with a flow rate of 400 uL/min. Ten mmoL ammonium acetate in water (A) and acetonitrile (B) were used as a mobile phase. Gradient elution were used as follows: 5% B (15 min), 5–55% B (15 min), 55–95% B (5 min), 95–5% B (5 min), 5% B (5 min). After the running, the gradient was set back to 5% B and the system was allowed to equilibrate. The injection volume was 10 µL and the detection wavelength was 280 nm. The ESI source was used and operated in positive ion mode. Typical operating conditions were as follows: drying gas (N_2) temperature of 350 °C, 5 L/min drying gas flow, 10 psi nebulizer gas (N_2) pressure, and 4500 V of capillary voltage. Data were acquired with a smart target of 30,000 and a max accumulation time of 200 ms. Full-scan MS spectra were obtained by scanning from 100 to 1300 m/z.

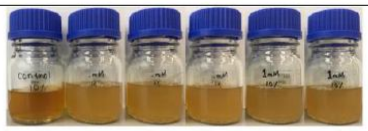
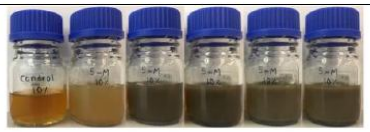
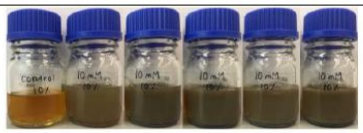
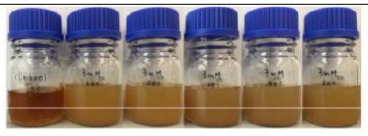
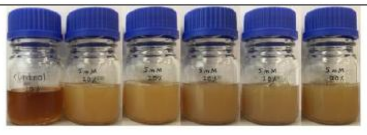
III. RESULT AND DISCUSSION

A. Colour Changes

Fig. 1 showed that among all concentration, only solution with concentration of 10 % of C. roseus aqueous extract in 5 mM and 10 mM AgNO3 solution; and 20 % of C. roseus aqueous extract in 10 mM AgNO3 solution shows colour changes from light yellow to brown-grayish solution after 24 h of incubation. The colour changes indicate the presence of AgNPs in the solution, resulting from the reduction of Ag ions into AgNPs when C. roseus aqueous extract was added to $AgNO_3$ solutions.

B. Ultraviolet-Visible (UV-Vis) Spectra Analysis

Further characterization was carried out using UV-Vis Spectroscopy to confirm the formation and stability of AgNPs. Fig. 2 show the highest absorbance peak at concentration of 10 % of C. roseus aqueous extract in 5 mM AgNO3 solution at approximately 450 nm, indicating the formation of AgNPs. Reference [21] reported that the absorption band of AgNPs is in the range of 350 nm to 550 nm. This absorption band, known as surface plasmon resonance (SPR) indicates that C. roseus aqueous extract was able to synthesize silver nanoparticles at the particular concentration. Thus, this concentration was chosen for further characterization.

Concentration	Colour changes (0, 2, 4, 22, 24 h)
1mM 10%	
3mM 10%	
5mM 10%	
10mM 10%	
1mM 20%	
3mM 20%	
5mM 20%	

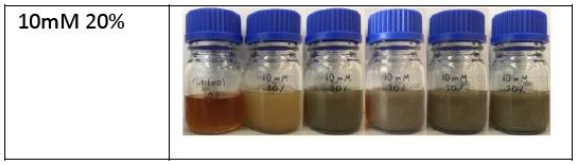


Fig. 1: Colour changes at 0, 2, 4, 22 and 24 h of incubation after the addition of 10 % and 20 % of C. roseus aqueous extract into 1, 3, 5 and 10 mM of $AgNO_3$ solution compared to 10 % and 20 % of C. roseus aqueous extract control.

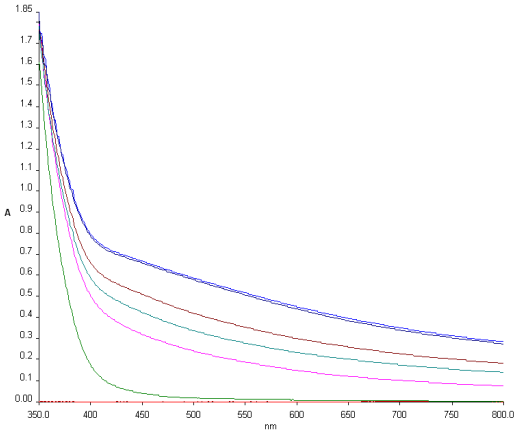


Fig. 2: XRD analysis of C. roseus-Agnps using 5mM: 10% ratio.

C. Transmission Electron Microscope (TEM)

The size and shape of AgNPs were observed by using TEM. AgNPs were harvested from the solution with the concentration of 10% (v/w) C. roseus aqueous extract in 5 mM AgNO3 solution. Fig 3 confirmed that the particles size of the AgNPs produced were in the range of 20 nm to 50 nm, with average diameter of 31 nm. AgNPs observed were in spherical shape.

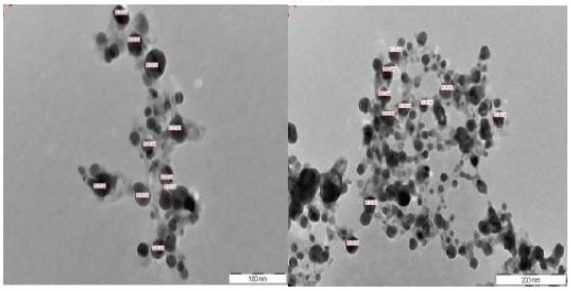


Fig. 3: Transmission electron microscopy (TEM) of C. roseus-Agnps using 5mM: 10% ratio.

D. X-ray Diffraction (XRD) Analysis

XRD was carried out to determine the crystal structure of AgNPs. From Fig. 4, distinct diffraction peaks at 2θ values were indexed with the planes 111, 200, 220, 311, 222, 400, 331, 420 and 422, which can be assigned to face-centered cubic silver. The well resolved and intense XRD pattern clearly shows that the AgNPs formed by the reduction of Ag^+ ions using C. roseus aqueous extract are crystalline in nature with face-centered cubic structure.

E. Zeta Potential Analysis

Zeta potential analysis was performed to gain further insights into the stability of the obtained colloidal AgNPs. From Fig 5, the zeta potential measurement of AgNPs exhibited at -16.8 mV, indicates that these AgNPs are highly stable due to the capping of biomolecules present in the C. roseus aqueous extract [22]. The capping of biomolecules was further confirmed using FTIR analysis.

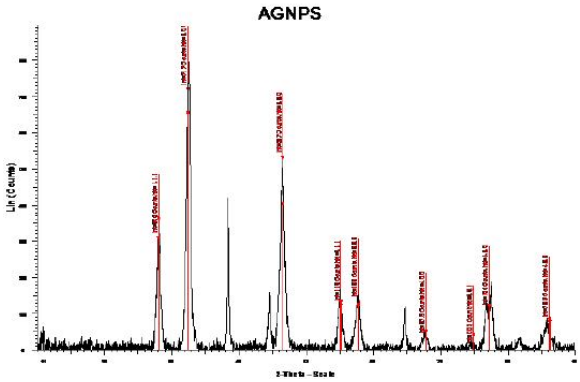


Fig. 4: XRD analysis of C. roseus-Agnps using 5mM: 10% ratio.

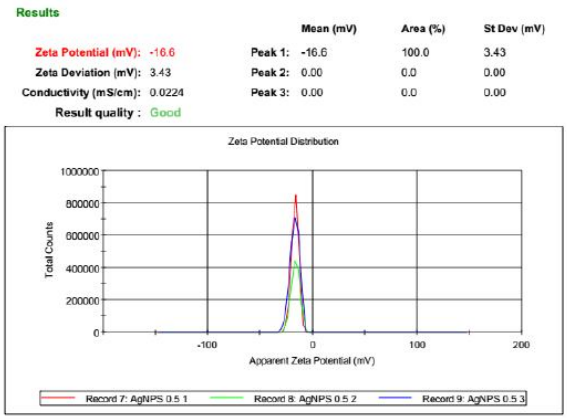


Fig. 5: Zeta potential analysis of C. roseus-Agnps using 5mM: 10% ratio.

F. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was performed to find the possible functional groups and biomolecules for capping and efficient stabilization of the synthesized AgNPs. In Fig. 6, the FTIR spectrum of C. roseus aqueous extract showed the absorption band at 3210.83 cm^{-1} (C-H stretch), 2934.11 (C-H bond), 1578.15 (N=O stretch), 1388.76 & 1314.89 (N=O bend), 1119.29 (C-O bond) and 729.94 (C-Cl bond). While, the FTIR spectrum of C. roseus-Agnps showed the absorption band at 2925.01 & 2924.97 (C-H bond), 1622.93 (C-C=C symmetric stretch), 1383.19 & 1384.13 (N-O bend), 1037.92/1038.76/1238.3/1117.2 (C-O bond), 3169.4 (O-H bond), 774.59 & 691.53 (C-Cl bond). The characteristics of C-H bond, C-O bond, C-Cl bond, N=O bend and C-N bond stretching vibrations are common in both C. roseus aqueous extract and C.

roseus-Agnps indicating that these biomolecules were involved in the reduction and capping of silver nanoparticles [23]. C-H bond stretching mode found in alkane, C-O bond stretching mode are the carbonyl functional groups of ester and alcohols, C-Cl bond are alkyl halides, N=O bend represents nitro groups and C-N stretching bands are available in amine stretch of proteins and amino acids, present in the leaves extract of C. roseus [22]. The presence of active functional groups in leaves extract refused in swift reduction of silver ions to silver nanoparticles. This further confirmed the silver nanoparticles obtained in present study might be surrounded by proteins having similar functional groups as the extract, such as amide and alkanes.

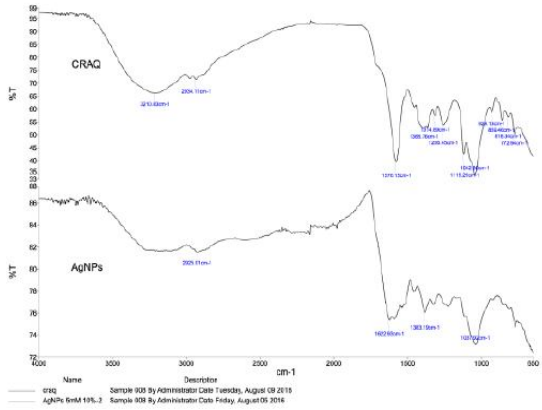


Figure 6: Fourier transform infrared spectroscopy (FTIR) of C. roseus aqueous extract and C. roseus-Agnps using 5mM: 10% ratio.

G. Liquid Chromatography/ Time-of-Flight Ion Trap Mass Spectrometry (LC/TOF-MS)

Analyses were carried out for both type of C. roseus aqueous extract samples, either prepared in 40 ˚C waterbath or boiled to study the presence of active compound in the extract. LC method was optimized to best chromatographic peak shapes. Various gradients of mobile phases at a flow rate of 400 µL/min were used for optimizing the elution conditions in order to achieve the reliable quantification of these alkaloids. From Fig. 7, both samples exhibit same active compounds despite the difference in preparation methods. It was observed that several peaks were composed of three components, but most peaks had symmetric peak shape and good resolution. LC/TOF-MS confirmed the presence of indole alkaloids which were lochnerine m/z 325, serpentine m/z 349, catharosine m/z 385, vincristine m/z 825, catharine m/z 823, ajmalicine m/z 353, vinleurosine m/z 809, and vindolicine m/z 925. Vindolinine, tabersonine and catharanthine exhibit identical protonated molecule ions of m/z 337 and were identified as isomers. Fig 8 shows the chemical structure of the identifed compounds. The identification of the compound in the extract was obtained by mass spectra, retention times, molecular weight (MW), and relative contents reported in literatures [24]-[29].

CONCLUSION

It has been concluded that the extract of C. roseus leaves is capable of producing AgNPs extracellularly by rapid reduction of silver ions, thus providing the evidence for developing large scale commercial production of value-added products for biomedical or nanotechnology-based industry. This further strengthens the alternative usage of green synthesis as one of the rapid, reliable, and best routes for the synthesis of silver nanoparticles (AgNPs). Uv-Vis spectra analysis indicated that AgNPs at concentration of 5 mM $AgNO_3$ and 10% extract has produced the highest surface plasmon resonance peak at approximately 450 nm. The shape and size determined by TEM indicates that the AgNPs sizes were in the range of 20 to 50 nm (average size of 31 nm with spherical shape). The XRD analysis confirmed the face-centered cubic structure of AgNPs. Zeta potential analysis exhibited the value at -16.8 mV, suggesting high stability of AgNPs due to the capping of biomolecules present in the C. roseus aqueous extract. FTIR spectrum of C. roseus aqueous extract showed the absorption band at 3210.83 cm^{-1} (C-H stretch), 2934.11 (C-H bond), 1578.15 (N=O stretch), 1388.76 & 1314.89 (N=O bend), 1119.29 (C-O bond) and 729.94 (C-Cl bond). While, the FTIR spectrum of C. roseus-Agnps showed the absorption band at 2925.01 & 2924.97 (C-H bond), 1622.93 (C-C=C symmetric stretch), 1383.19 & 1384.13 (N-O bend), 1037.92/1038.76/1238.3/1117.2 (C-O bond), 3169.4 (O-H bond), 774.59 & 691.53 (C-Cl bond). LC/TOF-MS confirmed the presence of indole alkaloids which were vindolinine, tabersonine, catharanthine, serpentine, catharosine, vincristine, catharine, ajmalicine, vinleurosine, and vindolicine. The present findings have shown that the AgNPs have been successfully synthesised and contain anticancer compounds that responsible for its synthesis.

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DISCLOSURE

The authors report no conflict of interest in this work.

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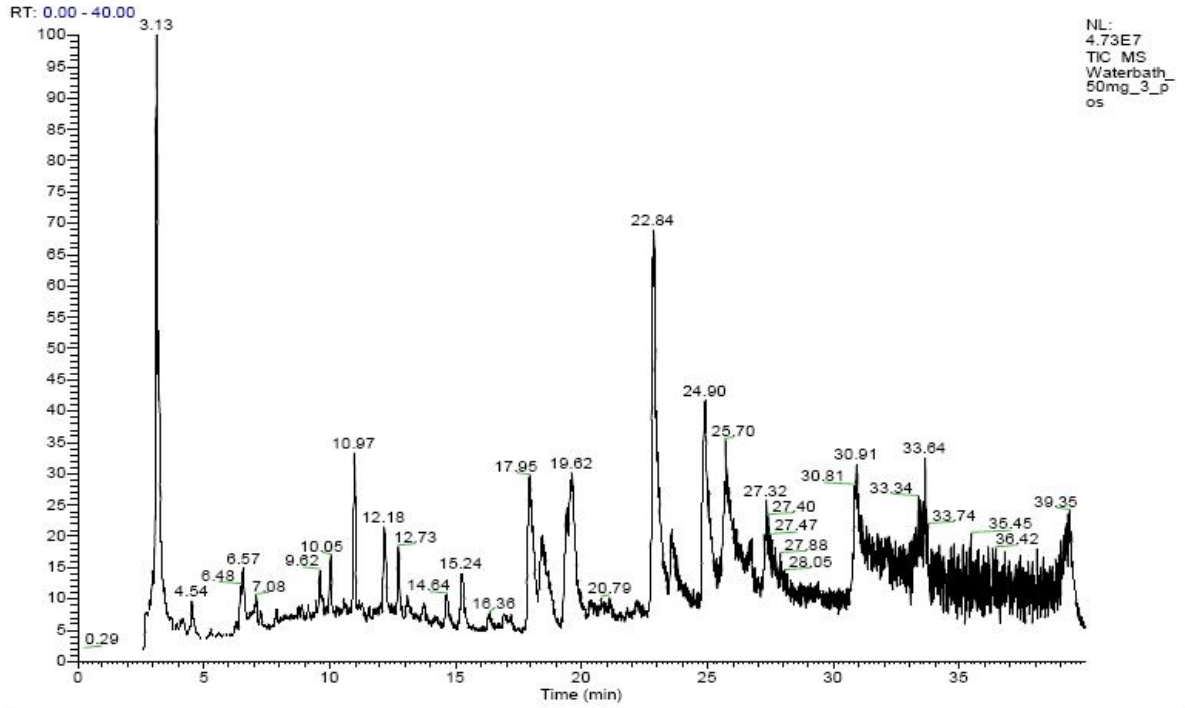


Figure 7 (a): Full-scan ESI-MS spectra of C. roseus aqueous extract, prepared in 40□ waterbath, in positive ion mode by direct loop injecting in ion trap mass.

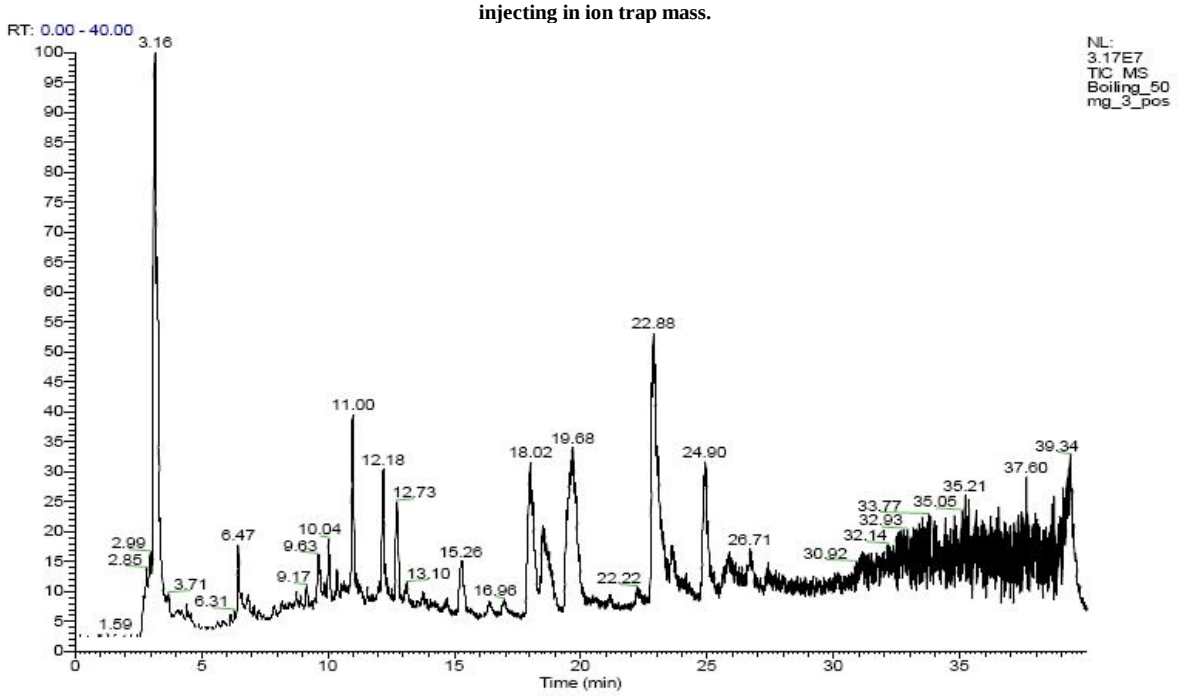


Figure 7 (b): Full-scan ESI-MS spectra of C. roseus aqueous extract, prepared by boiling, in positive ion mode by direct loop injecting in ion trap mass.Fig. 8: Chemical structure of identified compounds

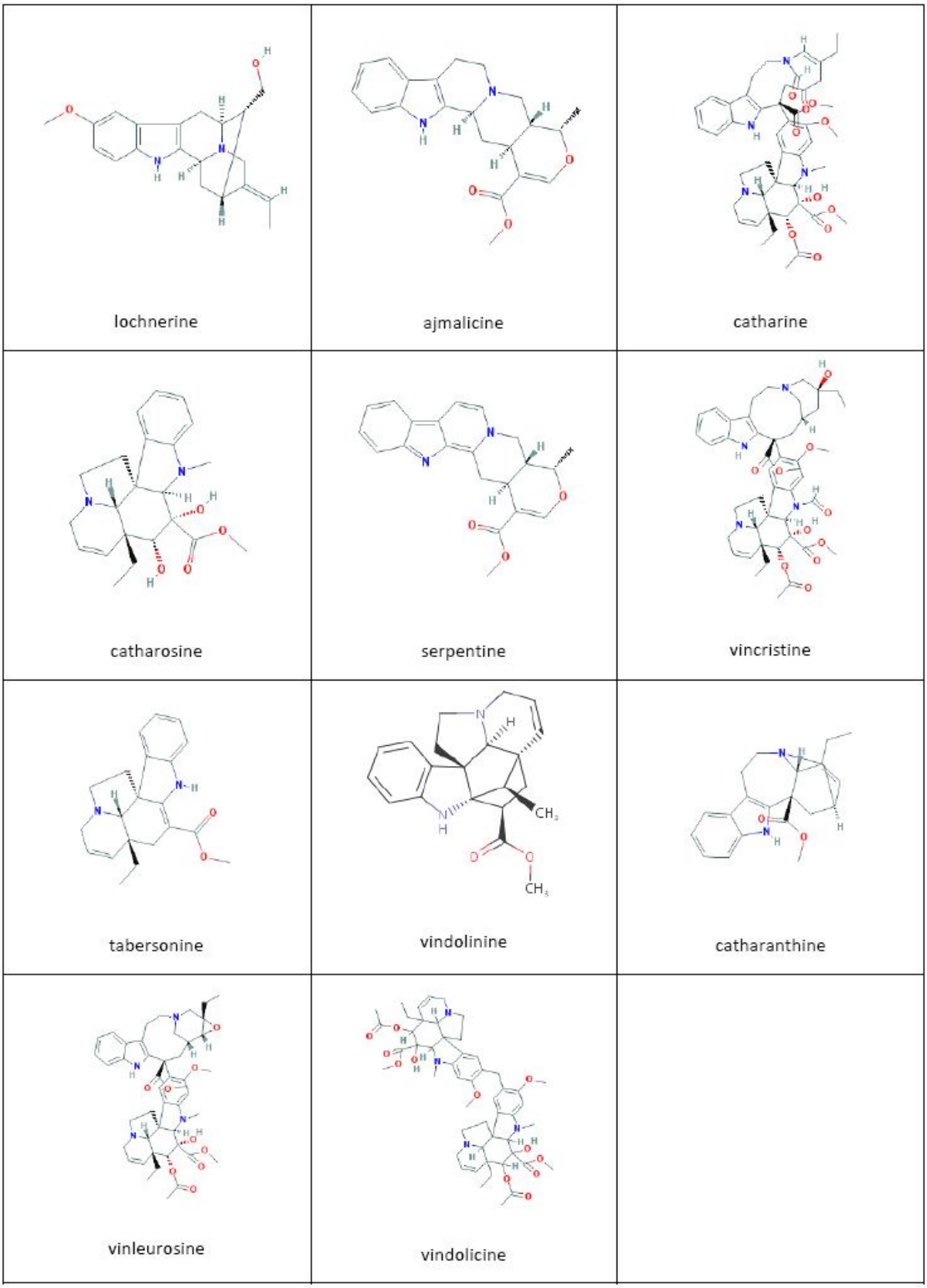


Fig. 8: Chemical structure of identified compounds