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Original Article

IN SITU ISOLATION AND CHARACTERIZATION OF NANO-USNIC ACID FOR MEDICAL APPLICATIONS

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ABSTRACT

Objective: Lichens, a unique division in the plant kingdom have been used in the treatment of diverse diseases in the past, by Native people of various countries. The medicinal utility of lichens has been connected to the presence of secondary metabolite compounds by scientific studies. Among a range of secondary metabolite compounds produced by Lichen, Usnic acid is the most extensively studied secondary metabolite, which possesses many biomedical activities like antiviral, antiprotozoal, antiproliferative, anti-inflammatory and analgesic.

Methods: We have optimized a protocol to isolate Nano Usnic acid *in situ* from the acetone extract of *Cladonia rangiferina* by Column Chromatography and Thin Layer Chromatography.

Results: The isolated Nano Usnic acid was characterized by various techniques and compared with standard usnic acid. The presence of Nano Usnic acid was confirmed by the UV-Visible Spectroscopy and FTIR Spectroscopy. The spherical shape and the nano size (60-120nm) of the isolated Usnic acid were corroborated with Particle Size Analysis and Scanning Electron Microscope.

Conclusion: Nano usnic acid may emerge as a potent therapeutic tool.

Keywords: Lichen, Chromatography, Nano usnic acid, UV-visible, FTIR.

INTRODUCTION

Since long time, natural biosources like plants, bacteria, fungus and lichens have been used as medicines [1]. Among them lichen have been reported to be the source of many medicinally valued phytochemicals such as depsides, depsidones, and usnic acid. Native Americans and Europeans used lichens in ancient medicine, in the treatment of pulmonary tuberculosis wounds and skin disorders. They have also been used in the treatment of diverse diseases like arthritis, alopecia, constipation, kidney diseases, leprosy, pharyngitis rabies, infection, cold, arthritis, fever, jaundice, constipation, convulsion, cough and tuberculosis.

Among the phytochemicals found in lichens, Usnic Acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione] is unique. Usnic acid was first isolated by Knob in 1844. Lichens such as *Cladonia, Usnea, Alectoria, Lecanora, Ramalina* and *Evernia* species are rich in Usnic Acid. Usnic acid has been reported to exhibit anti-microbial, antiviral, antiprotozoal, antiproliferative, anti-inflammatory and analgesic properties [2-5]. Therefore, Usnic acid can be used as a therapeutic agent for a broad spectrum of diseases and disorders. However the pharmacological and therapeutic activities of many therapeutic agents are decreased due to its non-polar nature, poor solubility and less absorption ability. These disadvantages can however be circumvented and the pharmacological activities be improved via nanotechnology. That is, by using nanosized compounds for high therapeutic efficacy. Moreover, Nanosized drugs possess the desirable characteristics such as high efficacy at low dose, less side effects, more dispersibility, higher saturation solubility, rapid dissolution, enhanced adhesion to biological surfaces, rapid onset of therapeutic action and improved bioavailability [6].

Therefore, in this study we optimized a method to isolate of nano usnic acid with medicinal value from *Cladonia rangiferina*.

MATERIALS AND METHODS

Collection and Identification of Species

Lichens were collected from coconut trees in a village in Kanyakumari district, Tamil Nadu and shade-dried. The Lichen sample was identified as *Cladonia rangiferina (L.) Nyl* of the family *Cladoniaceae* [7], by the botanist of Plant Anatomy Research Centre, West Tambaram, Chennai, India, based on the organoleptic macroscopic examination of the sample.

Isolation of Usnic Acid

The steps we optimized to isolate usnic acid are shown below and represented in the (Fig. 1).

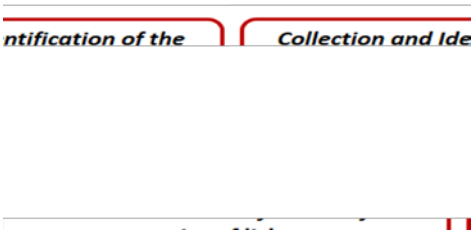

Fig. 1: Flowchart for *in situ* isolation of usnic acid

Preparation of Crude Lichen Extract

Crude Lichen Extract (CLE) was prepared as follows. 10g of the dried *Cladonia rangiferina* was taken in the round bottom flask and extracted with 300ml acetone at 70^{0}C for 48 hrs using the Soxhlet apparatus. The extract was concentrated by evaporating the solvent at room temperature.

Optimization of Solvent for Column Chromatography

To optimize the solvent mixture for fractionation and isolation of usnic acid, the CLE was subjected to thin layer chromatography

using five different solvent systems (Toluene: Acetic acid (9:1), Chloroform: Acetone (9:1), Chloroform: Acetone: Glacial Acetic acid (9:1:1), Methanol: Acetic acid: Acetone (9:1:1) and Chloroform: Acetone (7:3)). The fractionation patterns of the extract produced by different solvent mixture are shown in the (Fig. 2a). Among the five solvent systems used, Choloroform: Acetone (7:3) produced sharp bands with good resolution as shown in the (Fig. 2b). Therefore, Choloroform: Acetone (7:3) hereafter mentioned as OS (Optimal Solvent) is used for the isolation of usnic acid from CLE.

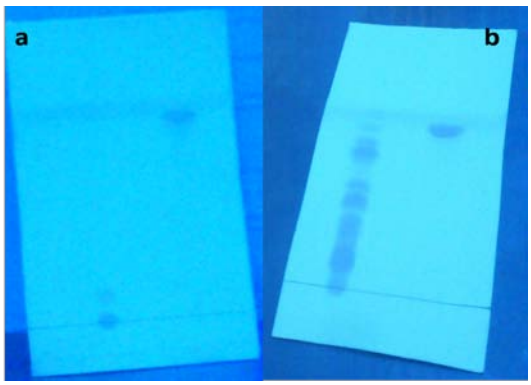

Fig. 2: Photograph of TLC a) different solvent systems b) Chloroform: Acetone

Isolation of Usnic acid using OS

The CLE is fractionated using the silica gel column of mesh size 100-200 and eluted using the OS. The fraction was collected at an interval of 10 mins. The fractions were represented arbitrarily as F1, F2, F3, F4, F5, F6, F7 and F8. Together, the fractions from column 1 were represented as C1F1, C1F2, C1F3, C1F4, C1F5, C1F6, C1F7 and C1F8. The fractions (C1F1 to C1F8) were subjected to TLC along with Standard Usnic Acid (SUA). The fractions that produced a sharp band equivalent to that of SUA were identified.

The corresponding fractions were again passed through the column and eluted as described above. The procedure was repeated till we get the fraction that gives only usnic acid bands comparable with that of SUA.

Characterization of Usnic acid

SUA, C5F4 and CLE were solubilized in acetone and characterized using various techniques. Absorption properties of SUA, C5F4 and CLE were analyzed using UV-Visible Spectrometer (T90 PG Instrument). The FTIR spectrum of the compounds was recorded on Perkin Elmer Spectrum Fourier Transform Infrared spectrophotometer. The average size and surface morphology were determined by Particle Size Analyzer (Malvern Zetasizer) and Scanning Electron Microscope (TESCAN).

RESULTS

The CLE was passed through the column and eluted at ten minutes interval to collect 8 different fractions. (Fig. 3a & 3b represents the column before and after elution). The fractions were denoted as C1F1 to C1F8, where C represents the column and F represents the corresponding fractions. C1F1 to C1F8 were further separated in TLC along with SUA. The Rf value of the bands obtained were compared with the Rf value of the SUA. Band with the Rf value equal to that of SUA (matching bands) were located. We identified sharp matching bands in the track of C1F1, C1F2 and C1F3 (Fig. 4-1(a-i)), hence, these three fractions were further fractionated in a second column (C2). We isolated 8 fractions named as C2F1 to C2F8 and chromatographed in TLC using SUA as described above. The TLC plates showed matching bands in C2F1 to C2F4 (Fig. 4-2 (a-i)). These 5 fractions were further fractionated in a third column (C3) and 8 fractions were collected (C3F1 to C3F8) and subjected to TLC. The TLC plates were then observed for matching bands as before. We noticed that C3F2, C3F3, C3F4, C3F5 and C3F6 showed sharp matching bands (Fig. 4-3 (a-i)). C3F2, C3F3, C3F4, C3F5 and C3F6 were passed into a fourth column (C4). As usual, 8 fractions, say, C4F1 to C4F8 were eluted and further resolved by TLC. C4F1 to C4F6 produced distinct matching bands (Fig. 4-4 (a-i)). Finally, C4F1 to C4F6 fractions were subjected to column chromatography in a fifth column (C5) and the fractions eluted (C5F1 to C5F8) for subsequent TLC fractionation. The thin layer chromatogram showed matching bands corresponding to C5F1 to C5F6 (Fig. 4-5 (a-i)). Among them, C5F4 matching band was sharper and denser than the other four. Therefore, we have selected C5F4 and its corresponding matching band for further characterization.

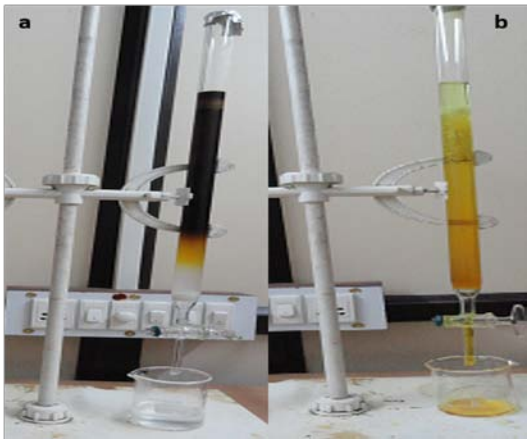

Fig. 3: Column chromatogram a) before elution b) after elution

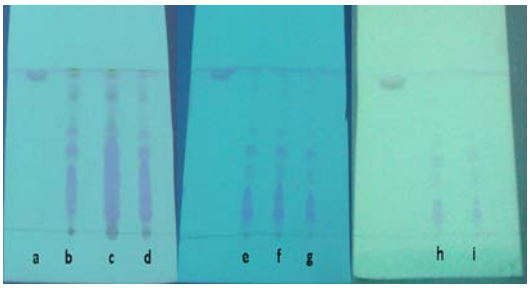

Fig. 4-1: TLC of chromatographic fractions collected from coloumn 1, a)SUA, b) C1F1, c) C1F2, d) C1F3, e) C1F4, f) C1F5, g) C1F6, h) C1F7, i) C1F8

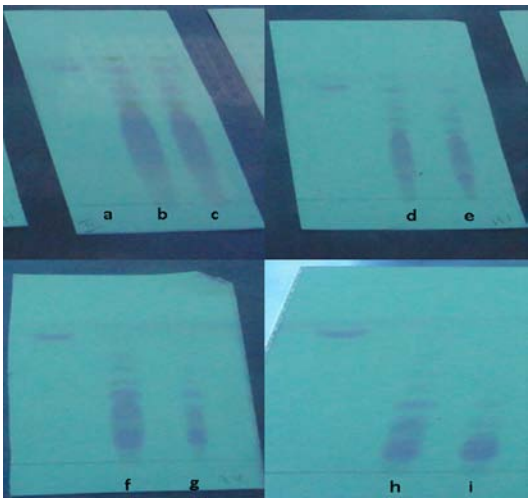

Fig. 4-2: TLC of chromatographic fractions collected from coloumn 2, a) SUA, b) C2F1, c) C2F2, d) C2F3, e) C2F4, f) C2F5, g) C2F6, h) C2F7, i) C2F8

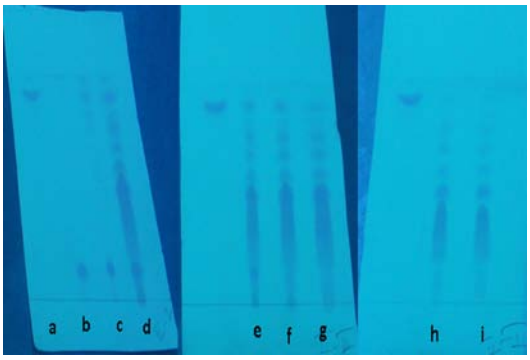

Fig. 4-3: TLC of chromatographic fractions collected from coloumn 3, a) SUA, b) C3F1, c) C3F2, d) C3F3, e) C3F4, f) C3F5, g) C3F6, h) C3F7, i) C3F8

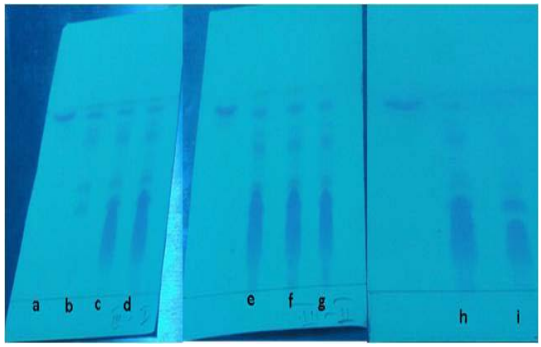

Fig. 4-4: TLC of chromatographic fractions collected from coloumn 4, a) SUA, b) C4F1, c) C4F2, d) C4F3, e) C4F4, f) C4F5, g) C4F6, h) C4F7, i) C4F8

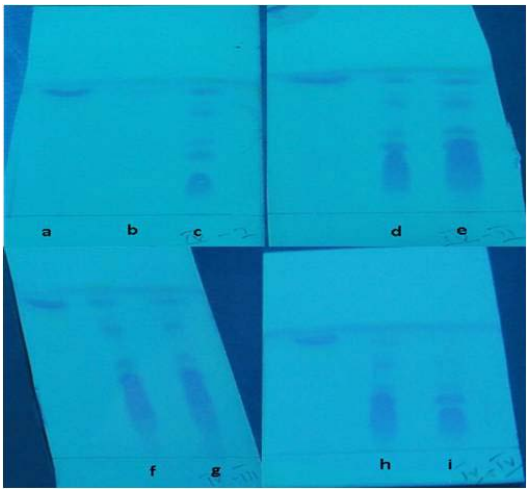

Fig. 4-5: TLC of chromatographic fractions collected from coloumn 5, a) SUA, b) C5F1, c) C5F2, d) C5F3, e) C5F4, f) C5F5, g) C5F6, h) C5F7, i) C5F8

On the whole, it was interesting to note that the number and the resolution of the non-matching bands decreased gradually from C1 to C5. The similarities in Rf values between the SUA band and C5F4 band in the TLC is the initial indicator for the presence of usnic acid in the fraction. As the bands produced by the C5F4 are sharper and denser with high resolution. We could further justify that the product obtained by this isolation protocol is usnic acid only. Still, we need to confirm that the usnic acid we isolated is in nano scale and could be used for therapeutic applications. Therefore we scraped the band in the TLC of C5F4 fraction and dissolved in acetone. The mixture was centrifuged and the supernatant containing usnic acid of the lichen extract (ULE) is characterized along with SUA and C5F4 fraction.

Characterization Studies

The size and morphology of the SUA, C5F4 and ULE were confirmed by SEM. (Fig. 5) displays the SEM images of SUA, C5F4 and ULE. We could observe uniformly sized spherical particles with a mean size of 100 nm. The nano usnic acid particles were distributed uniformly with less agglomeration.

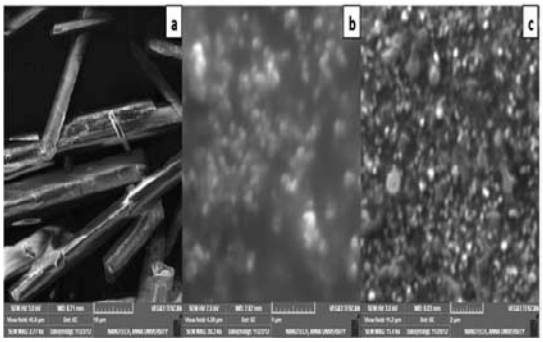

Fig. 5: SEM images of a) SUA, b) C5F4, c) ULE

The SEM result clearly indicates the presence of nano-usnic acid. We could also justify that the isolation protocol we described here is optimal for the preparation of nano usnic acid. In addition, absence of agglomeration indicates that the nano usnic acid is dispersed and stable, which may enhance its bioavailability and pharmacological activity. UV-Visible spectra obtained for SUA, C5F4 and ULE are shown in the (Fig. 6).

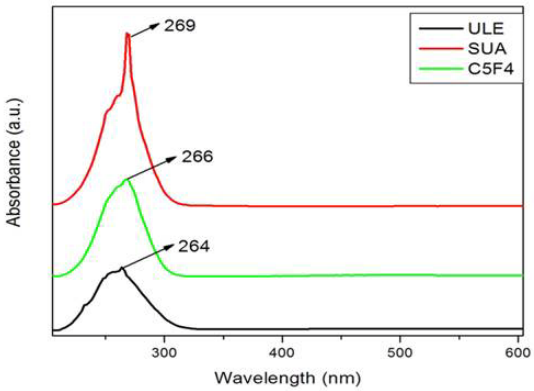

Fig. 6: UV-Vis Absorption Spectra of SUA, C5F4 and ULE

SUA shows absorption peak at 269nm. The chromatographic fraction C5F4 shows peak at 264nm indicating the presence of usnic acid. ULE displays a single peak in the chromatogram at 266nm. From the spectral data, it is evident that the C5F4 chromatographic fraction is usnic acid and the corresponding bands obtained from the TLC plates.

The IR spectrum of usnic acid presents a characteristic band of the conjugated ketone cyclic group at 1645 cm^{-1}. Peak at 3446cm^{-1}, 3303cm^{-1}, 3124cm^{-1} is due to stretching of O-H bond in Ar-OH intramolecular hydrogen bond. Alkane groups (-CH_3) shows peaks at 2921cm^{-1} and 2854cm^{-1} due to C-H bond stretching. Similarly, -C=C- group shows peak at 1645cm^{-1} which indicates the presence of aryl group. The conjugation, electron donation of the constituent rings, and possible hydrogen bonds contributed to the small wavelength of the aromatic methyl ketone at 1632 cm^{-1}. It is possible to assign the conjugated cyclic ketone group to the 1694

cm^{-1} band. In addition, the IR spectrum of usnic acid included a band of hydroxyl phenolic groups at 3150 cm^{-1} It is also possible to assign the anti-symmetric and symmetric stretching n(C-O-C) aryl alkyl ether bands at approximately 1277 and 1070 cm^{-1} respectively. FTIR pattern of C5F4 and ULE is similar to that of SUA, thus confirming the presence of usnic acid in the C5F4 and ULE. FTIR spectra for the SUA, C5F4 and ULE are displayed in the (Fig. 7).

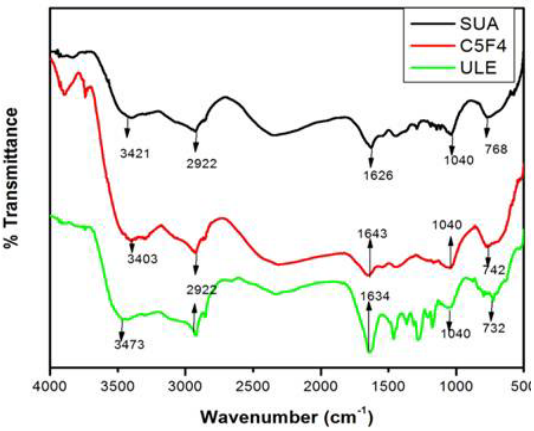

Fig. 7: FTIR Spectra of SUA, C5F4 and ULE

The size and distribution pattern of the nano usnic acid in the C5F4 and ULE band were reconfirmed by particle size analysis. Particle size data for SUA, C5F4 and ULE are shown in the (Fig. 8). The results indicate that the size of SUA was found to be 4300 nm, which may be due to its rod shape as revealed in SEM images as indicated in the (Fig. 5).

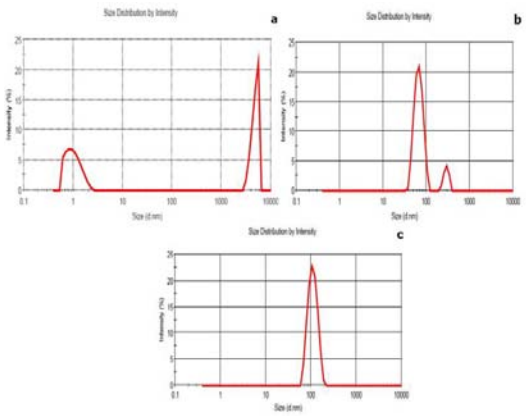

Fig. 8: Particle Size Data of Chromatographic fraction a) SUA, b) C5F4, c) ULE

The graphical data of C5F4 and ULE displayed single peaks with 100 % intensity at a size range of 60-120 nm indicating that both of them possess uniform size distribution. Thus we emphasize that the size of isolated usnic acid is in nano scale. This is the first report on the isolation and characterisation of nano usnic acid from lichens and our view is very well supported by the SEM and PSA.

DISCUSSION

For the first time we have isolated nano usnic acid from lichen extract by column chromatography and thin layer chromatography using an optimized and new solvent mixture Chloroform: Acetone (7:3). The presence of Usnic acid in the fractions from column Chromatography and TLC was confirmed using the standard usnic acid as a reference. UV-Visible spectrum and FTIR spectrum of standard and isolated usnic acid were similar, thus confirming that the isolated compound is usnic acid. Further, the blue shift observed with nano usnic acid indicates size reduction. This is in line with the previous reports which correlate the blue shift in the UV peak with the size diminution [8, 9]. The morphology of nano usnic acid, as analysed by SEM clearly reveal the spherical morphology. According to Chithrani *et al.*, spherical nanoparticles have better cellular entry could possess better biomedical acitivity [10]. Thus, we could suggest that the nano usnic acid may have better therapeutic efficacy than the bulk SUA which has rod morphology. The size of the nano usnic acid as revealed by SEM was around 100 nm, which is roughly equal to the size estimated using Particle Size Analysis (80-120nm). Several reports have highlighted the enhanced biomedical efficacy of particles with the size less than 200nm [11], when compared to the bulk counterparts. This is due to the unique features of nanoparticles such as enhanced cellular uptake, penetration and retention in the blood vessels [12]. As the isolated usnic acid also falls under nanoscale range, they may possess high surface to volume ratio, dispersibility, bioavailability and eventually may have better therapeutic efficacy. These findings reveal that the isolation protocol is valid for the production of therapeutic nano usnic acid.

Usnic acid possess antiprotozoal, antimicrobial, antiproliferative, anti-inflammatory, antitumor and gastroprotective and antioxidant activities [13-22]. Since nano-drugs have properties such as reduced dose and side effects, resistance to settling, higher saturation solubility, rapid dissolution, enhanced adhesion to biological surfaces, rapid onset of therapeutic action and improved bioavailability, nano usnic acid is expected to have better therapeutic effects. Previous report suggest that nanotization of bioactive compounds may enhance their solubility, dispersibility and bioavailability, which inturn might enhance their therapeutic efficacy [23]. We could therefore suggest that the nano usnic acid could emerge as an excellent therapeutic tool.

CONFLICT OF INTERESTS

Declared None

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