Original Article

Isolation, Purification and Structural Elucidation of N-Acetyl-5-Methoxytryptamine (Melatonin) From *Crataeva nurvala* Buch-Ham Stem Bark

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ABSTRACT

The objectives of current study were to isolate, purify and characterize bioactive tryptamine derivative from *Crataeva nurvala*, a well explored traditional Indian medicinal plant with historical evidence of efficacy in the treatment of neurological and antioxidant deficiency related disorders. In this study, chloroform fraction of ethanolic extract of *Crataeva nurvala* stem bark was analyzed using column chromatography (gradient elution technique) and thin layer chromatography (TLC) to isolate melatonin, a biogenic indoleamine compound. The structure of the isolated compound was determined by various spectrophotometric analysis like UV, IR, ^{13}C and ^{1}H NMR and mass spectroscopy. Mass spectrum of isolated compound showed a parent molecular ion (M^+) peak at m/z 233.2 gm/mol correspond to the molecular formula $C_{13}H_{16}N_2O_2$. In the ^{1}H NMR spectrum, singlet (s) at δ_H 3.79 corresponds to 3 H of OCH_3, multiplet (m) at δ_H 6.7, 7.0, 7.7 denotes 4 Ar. Protons, singlet (s) at δ_H 1.9 assigned for 3 H of CH_3CO, triplet (t) at δ_H 2.8 corresponds to 2 H of N-CH_2, multiplet (m) at δ_H 3.27 denotes 2 H of indolyl CH_2, singlet (s) at δ_H 10.41 assigned for 1 H of NH indole and singlet (s) at δ_H 8.01 corresponds to 1 H, NH of secondary amide. ^{13}C NMR showed presence of total 13 carbon atom. Based on physical and spectral characteristics, the isolated compound was identified and reported for the first time as N-acetyl-5-methoxy tryptamine (Melatonin).

Keywords: *Crataeva nurvala,* Isolation, Melatonin.

INTRODUCTION

Melatonin, a biogenic indoleamine was first isolated from the bovine pineal gland and identified as N-acetyl-5-methoxy-tryptamine by Lerner and co-workers[1]. In mammals, melatonin plays a key role to regulate circadian rhythm with highest levels during scotophase and baseline levels during the photoperiod[2]. This molecule has been linked to a number of the physiological and pathophysiological functions including prevention of ischemia-reperfusion damages, relief of chronic pain, enhancement of immunity, antibacterial and oncostatic effects, treatment of the neurological disorders, anti-inflammatory, and anti-oxidative properties[3-8].

Since the identification of melatonin in plants by Hattori *et al*., several reports have published and opened up a new area in the field of plant derived melatonin i.e. phytomelatonin[9]. Phytomelatonin is biosynthesized in plants from tryptophan precursor[10]. Majority of the herbs containing high levels of melatonin have been used traditionally to treat neurological disorders associated with the generation of free radicals which might be associated with its potent antioxidant activity[11].

Crataeva nurvala (*C. nurvala*) Buch-Ham (Family: Capparidaceae) commonly known as Varuna, is a well explored traditional Indian medicinal plant used to treat various ailments in particular urolithiasis and neurological disorders[12,13]. Traditionally the stem bark is also used as stomachic, laxative, anthelmentic, expectorant and anti-pyretic[14]. Moreover, pharmacological study reveals multidirectional potentiality of *C. nurvala* extract and its active principle, particularly lupeol as diuretic, anti-inflammatory, antioxidant, cardio-protective, hepato-protective, lithonotriptic, anti-rheumatic, anti-periodic, contraceptive, anti-protozoal, rubifacient and vesicant[15]. Since no study has ever conducted to isolate tryptamine base from the stem bark of *C. nurvala*, the present study was designed to isolate and characterize melatonin from chloroform fraction of stem bark of *C. nurvala*.

MATERIALS AND METHODS

Collection and authentication of plant material

The stem bark of *C. nurvala* was collected from the stream sides of Westernghat, India and authenticated by Dr. K.V. Nagalakshamma, Professor and Head, Department of Biotechnology (UG) of St. Aloysius College, Mangalore, India. The herbarium (voucher specimen no. NGSMIPS/Hb-04/2011) was preserved in the institutional department.

Extraction and fractionation

1 kg coarsely powdered raw material of *C. nurvala* stem bark was extracted by cold maceration with ethanol and concentrated through rotary flash evaporator[16]. The yield was found to be 17 % w/w. The concentrated ethanolic extract (60 gm) was defatted with petroleum ether (4 x 100 ml) and fractionated with chloroform (4 x 100 ml) successively to afford chloroform soluble light brownish residue (14 gm). The chloroform soluble fraction was subjected for isolation of bioactive phytomelatonin. A flow chart of detailed method of extraction and fractionation is given in figure 1.

Isolation and purification of active compound

Chloroform fraction (10 gm) was subjected to purification by silica gel column chromatography using chloroform: ethyl acetate solvent system with increased order of polarity (from 0:100 to 100:0, v/v)[17]. The

progress of separation was monitored by TLC (silica gel G 60 F254 plates, Merck). The eluent was collected into twelve different fractions. Fraction-IV was subjected for re-chromatography where eluting with chloroform: ethyl acetate (7:3) resulted a crude amorphous yellowish white substance. After being washed with methanol, it was converted to colour less crystalline substance (20 mg). The crystals were further analyzed spectrophotometrically to elucidate the structure. TLC chromatogram developed with methanol: chloroform (2:8) was homogenous with R_f 0.58. Further, the chemical nature of the compound was evaluated by standard qualitative phytochemical screening methods.

Qualitative analysis

2 ml of 2 N HCl was added to 10 ml aqueous solution of isolated compound, the solution was heated for 10 min, cooled and subjected for following tests.

Dragendorff's test

To 2 ml of the above solution few drops of Dragendorff's reagent (potassium bismuth iodide solution) were added. Orange-brown precipitate was observed[18].

Mayers test

To 2 ml of the above solution few drops of few drops of Mayer's reagent (potassium mercuric iodide solution) were added. Cream coloured precipitate was observed[18].

Hager's test

To 2 ml of the above solution few drops of few drops of Hager's reagent (saturated solution of picric acid) were added. Yellow precipitate was observed[18].

Wagner's test

To 2 ml of the above solution few drops of few drops of Wagner's reagent (iodine in potassium iodide solution) were added. Reddish brown precipitate was observed[18].

Structural characterization of compounds

The UV spectra were recorded in the wavelength range 200-800 nm with Shimadzu UV-1700 Pharmac-spec UV-Vis spectrophotometer (Japan). The signals were acquired four times and the mean signals were taken as the best value of the UV spectra. Before every measurement the blank spectrum was also recorded, and automatically subtracted from the sample spectrum by the instrument software using the signal background ratio.

The IR spectra were recorded in the wave number range 400–4000/cm with a resolving power of 0.5/cm on an Alpha-Bruker IR spectrophotometer (Karlsruhe, Germany) from CH_2Cl_2 sample solution. The signals were acquired four times and the mean signals were taken as the best value of the FT-IR spectra. Before every measurement the blank spectrum was also recorded, and automatically subtracted from the sample spectrum by the instrument software using the signal background ratio.

^{1}H- and ^{13}C-NMR spectras were recorded on a Bruker Advance II 400 NMR spectrophotometer (Karlsruhe, Germany) in deuterochloroform solutions. ^{1}H-NMR chemical shifts are given in ppm form using tetramethylsilane (TMS) used as internal reference and ^{13}C-NMR chemical shifts (in ppm) are given from DMSO and were taken from fully decoupled spectra. ^{1}H-^{1}H COSY, experiment was performed with the usual pulse-sequence and data processing was obtained with standard software.

Low resolution and HR electron ionization (EI) MS were recorded by the Waters Q-TOF (Micromass, Altrincham, UK) and mass spectrometer connected with a GC system HP 6890 series (Hewlett Packard, Palo Alto, CA, USA). LR-EI-MS (resolution power 1500) and HR-EI-MS (resolution

power 8000, 10% resolution valley definition) were performed under the following experimental conditions: electron beam energy 70 eV; source temperature 210^0C; source pressure 10^-7 Torr; trap current 250 µA; emission current 2.3 µA; accelerating voltage 8.0 kV. Accurate mass measurements (± 10 ppm) were determined by HR-EI-MS using perfluorokerosene (PFK) as internal standard. Gas-chromatographic conditions were: injector temperature 290^0C; column AT^{TM}-5 (All tech, Deerfield, Fl, USA), film thickness 0.25 µm, length 30 m, ID 0.25 mm, carrier gas (helium) flow 1.0 mL/min, isotherm at 120^0C (5 min), ramp 120–240^0C (20^0C/min), isotherm 240^0C (9 min).

RESULTS

From the positive qualitative analysis, the isolated compound was assumed to be alkaloid in nature. The melting point was observed at 117.0^0C; UV spectroscopic analysis revealed the λ_{max} at 278 nm. IR spectrum showed presence of NH (amide) str. at 3726 cm^{-1}, NH-indole str. at 3432 cm^{-1}, CH_3/CH_2 str. at 2945 cm^{-1}, C=O str. at 1644 cm^{-1}, Ar. C=C str. at 1400 cm^{-1}, C-O str. at 1216 cm^{-1} , C-N (amine) str. at 1071 cm^{-1}.

The ^{1}H NMR spectrum (DMSO) showed a sharp singlet at δ_H 3.79 clearly indicates presence of Ar.OCH_3 group (Table: 1 and Figure 2). Aromatic protons were also accounted by the multiplet signals δ_H 6.7, 7.0, 7.7. Singlet signals appearing at δ_H 1.9 accounted for 3H of aliphatic $COCH_3$. The triplet signal appearing at δ_H 2.8 indicate the protons of N-CH_2. The multiplet appearing at δ_H 3.27 accounting for 2H can be assigned to indolyl-CH_2 of the alkaloid. Singlet signals appearing at δ_H 10.41 correspond to 1 H, -NH gr. of indole nucleus. The singlet appearing at δ_H 8.01 accounted for 1 H, -NH group of secondary amide. We have also performed the 2D COSY NMR spectrum which showed the expected cross peaks according to structure of melatonin (Figure 3). In fact, the cross peak attributed between the protons at 7.0 ppm and the multiplet near 3.0 ppm corresponds to the coupling of the indole NH and the H-8 of aromatic ring of melatonin.

Moreover, ^{13}C NMR spectrum revealed the presence of total 13 carbon atom (Figure 4); δ_{ppm} 152.92 at C-5 revealed presence of methoxy group (OCH_3) where as δ_{ppm} 55.20 at C-15 correspond to Ar.OCH_3 group. Indolyl-CH_2 peak at C-10 was observed at δ_{ppm} 29.07 and δ_{ppm} 25.03 at C-11 revealed presence of aliphatic N-CH_2 group. Further, δ_{ppm} 22.60 at C-14 denoted the presence of CH_3CO group. (Figure 5)

Mass spectroscopy revealed molecular ion peak at m/z=233.2, and base peak at m/z=255.1. Further, relative intensity of different fragments were summarized (Table 2; Figure 6). These assignments revealed the molecular formula of the isolated compound as $C_{13}H_{16}N_2O_2$. By comparing IR, TOF MS ES, ^{1}H & ^{13}C NMR data with existed literatures the isolated compound was assigned as N- acetyl-5-methoxy tryptamine (melatonin)[19-21].

DISCUSSION

C. nurvala, a potential traditional Indian medicinal plant is widely used to treat urolithiasis and neurological disorders mediated via free radical generation[22]. Hence, an attempt had been made to isolate bioactive phytoconstituents from stem bark of *C. nurvala* resulted in isolation of novel bioactive tryptamine derivative viz. N-acetyl-5-methoxy-tryptamine (melatonin).

In IR spectrum, a very broad band at 3726 cm^{-1} and moderately intense band at 3432 cm^{-1} reflects the presence of NH (amide) group and NH-indole group. Stretching and bending vibrations of in plane CH_3/CH_2 groups were noticed by intense band at 2945 cm^{-1} and medium intense band at 1644 cm^{-1}corresponds to C=O stretching.

In ^{1}H NMR spectrums, different peaks were observed at at δ 3.79 (s, 3 H, -OCH_3 gr.), δ 6.7, 7.0, 7.7 (m, 4 H, Ar. Protons), δ 1.9 (s, 3 H, -CH_3CO gr.), δ 2.8 (t, 2 H, N-CH_2 gr.), δ 3.27 (m, 2 H, indolyl-CH_2 gr.), δ 10.41 (s, 1 H, -NH gr. of indole), δ 8.01 (s, 1 H, -NH gr. of sec. amide) and revealed presence of total 16 protons. Moreover, ^{13}C NMR revealed presence of total 13 carbon atom. These assignments are in good agreement for the confirmation of melatonin structure.

Further, during isolation process wide range of phytoconstituents were observed in partially purified bands which indicate the necessity of further studies to isolate few more lead compounds. Moreover, this study will stimulate mechanism based pharmacological aspects of *C. nuvala* plant against various neurological disorders.

CONCLUSION

A new method of isolation for novel bioactive tryptamine alkaloid i.e. melatonin had been developed and reported for the first time which might be extremely suitable as marker compounds for standardization of commercial extract and herbal-preparation containing *C. nurvala*. In addition, these results will explore a new pre-clinical aspect to find the utility of *C. nurvala* against neurological disorders associated with the generation of free radicals like Alzheimer's.

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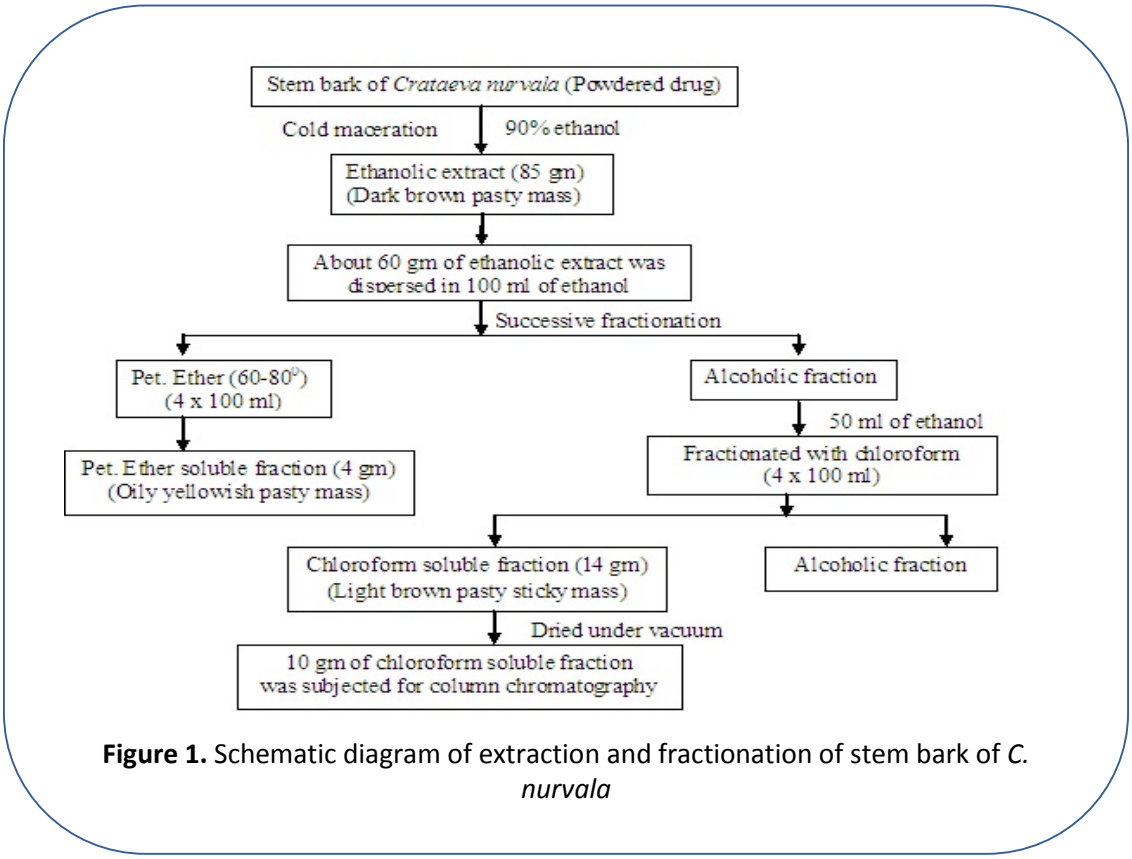

Figure 1. Schematic diagram of extraction and fractionation of stem bark of *C. nurvala*

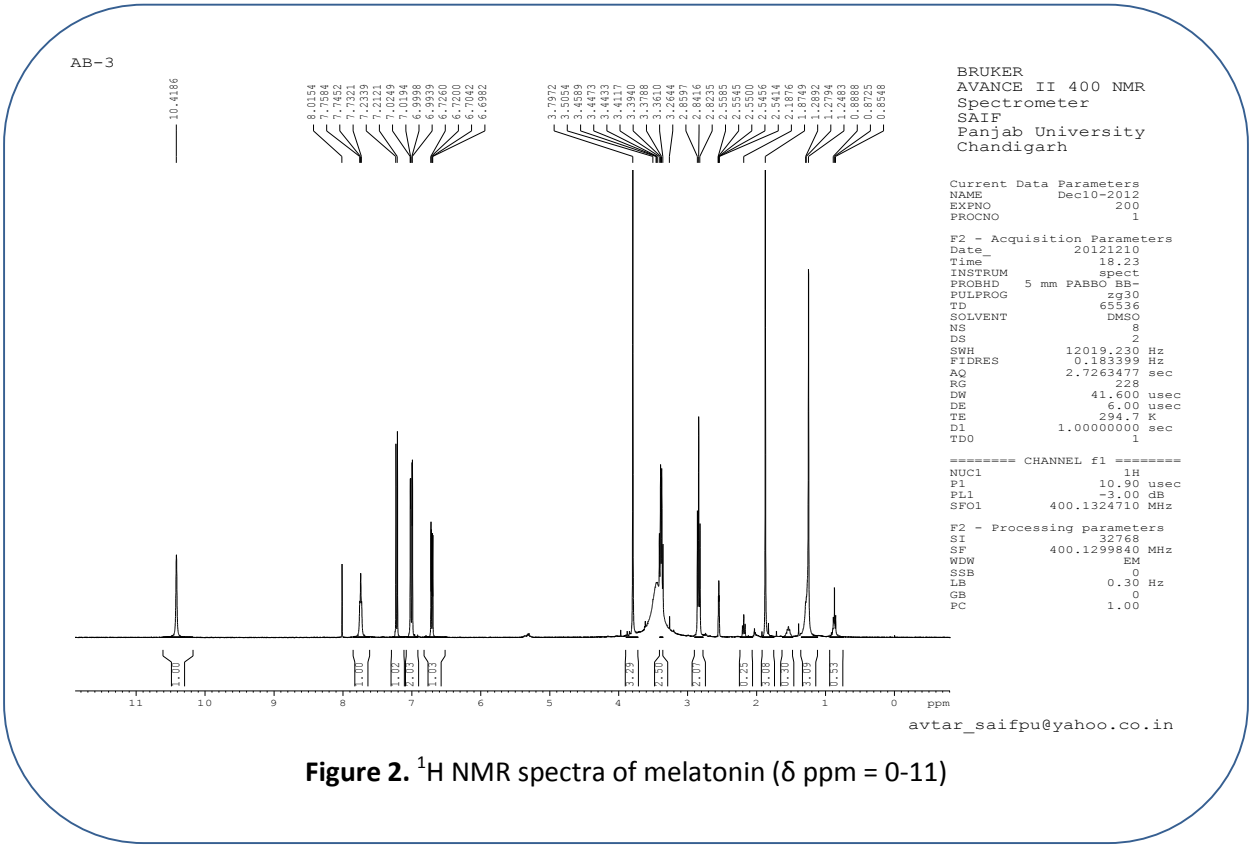

Figure 2. ^{1}H NMR spectra of melatonin (δ ppm = 0-11)

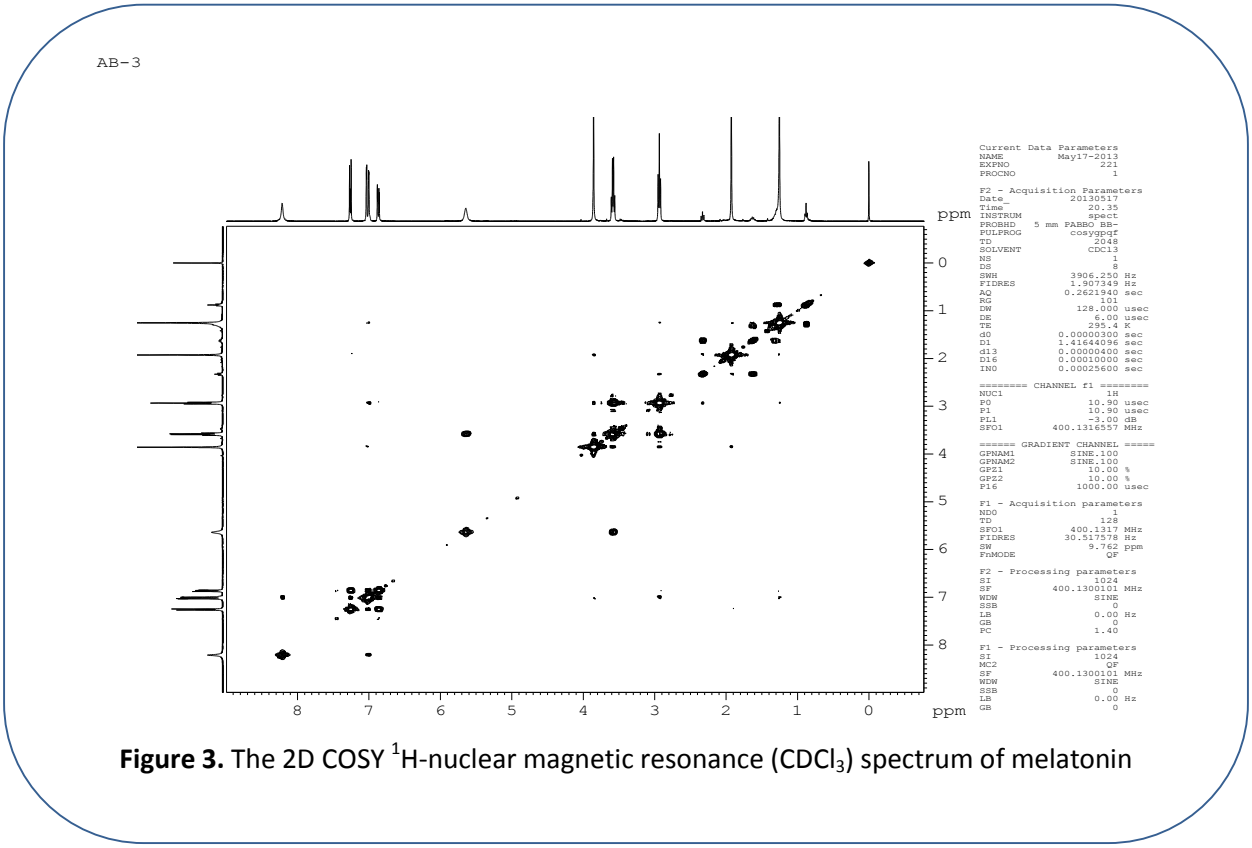

Figure 3. The 2D COSY ^{1}H-nuclear magnetic resonance ($CDCl_3$) spectrum of melatonin

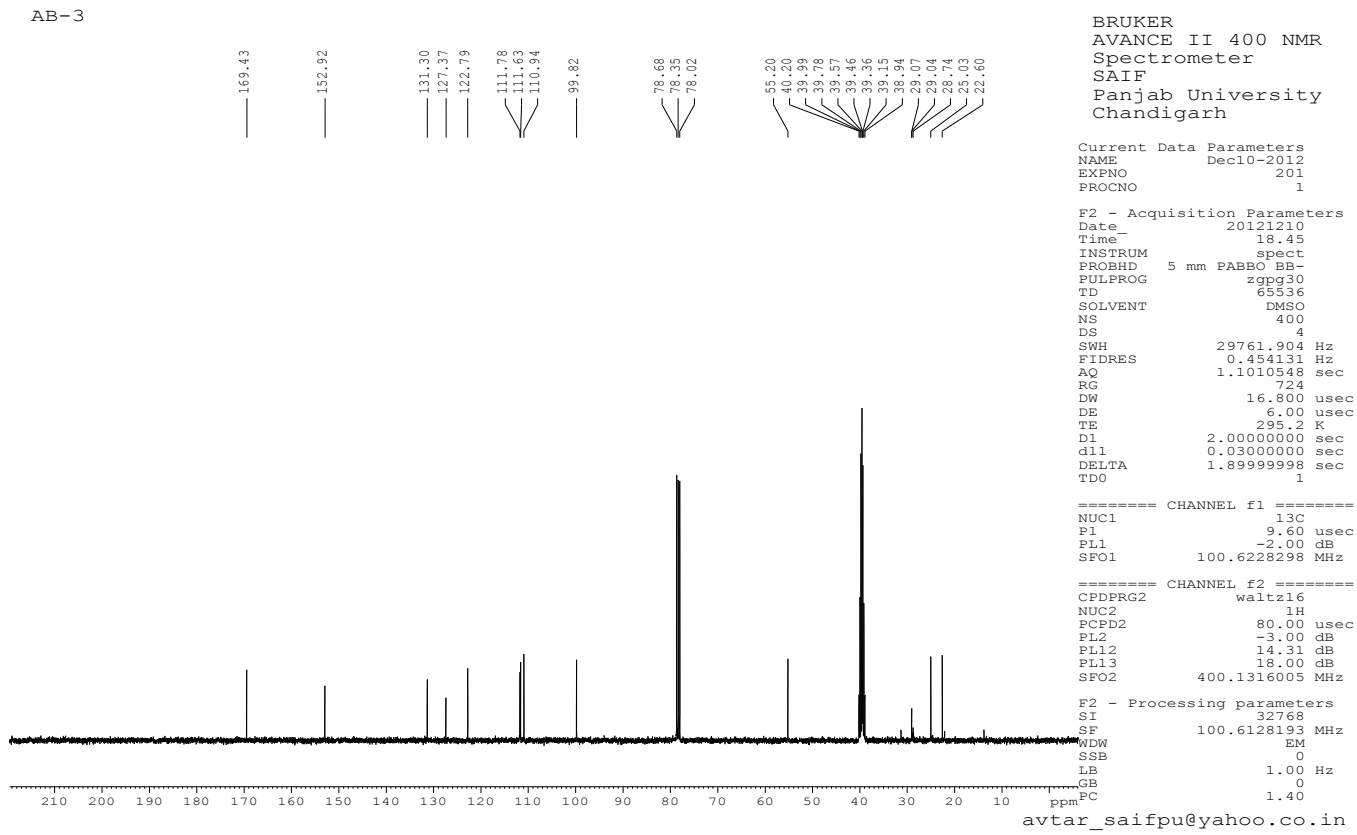

Figure 4. ^{13}C NMR spectra of melatonin (δ ppm = 0-200)

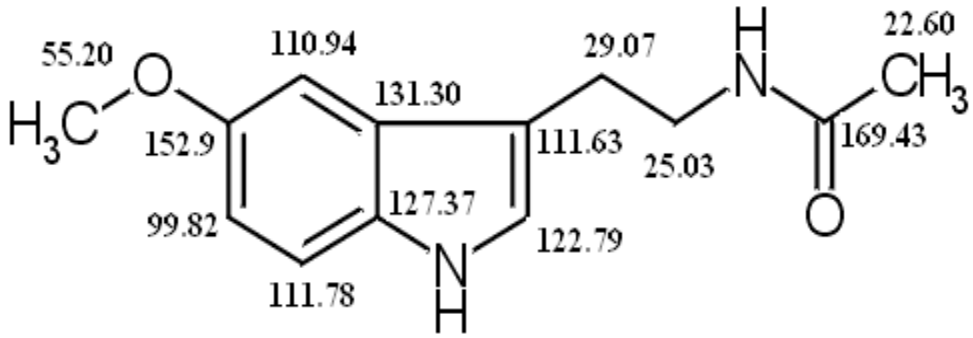

Figure 5. ^{13}C-NMR chemical shifts of N- acetyl-5-methoxy tryptamine (Melatonin)

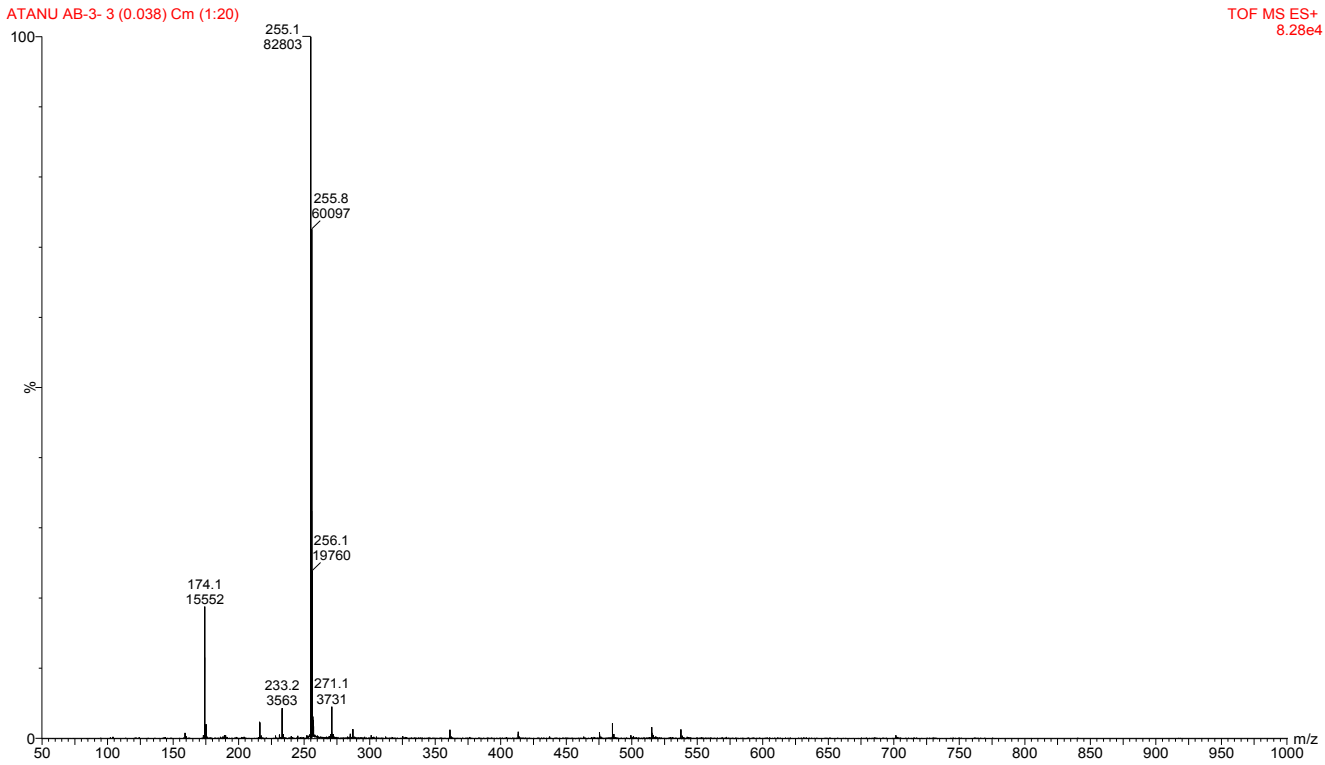

Figure 6. High resolution-electron ionization- mass spectra (70 eV) spectrum of melatonin

Table 1. The 1H-nuclear magnetic resonance data of melatonin, chemical shifts in ppm relative to the internal standard DMSO

δ (ppm)	Spin multiplicity	Integration	Comment
3.79	s	3 H	OCH_3
6.7, 7.0, 7.7	m	4 H	Ar. Protons
1.9	s	3 H	CH_3CO
2.8	t	2 H	N-CH_2
3.27	m	2 H	indolyl-CH_2
10.41	s	1 H	-NH gr. of indole
8.01	s	1 H	-NH gr. of sec. amide

s = singlet, d = doublet, t = triplet.

Table 2. Mass spectra data of isolated compound, fragmentation pattern

EIMS (m/z) (%)	233.2 [M^+], (8 %),
Relative intensity	174.1 (20 %), 255.1 (100 %), 255.8 (78 %), 256.1 (25 %), 271.1 (9 %)