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In vitro antimicrobial assay of an alkaloid isolated from the leaves of *Pterocarpus santalinus* L.F.

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Abstract

Due to the adverse side effects of extensive usage of synthetic drugs, people are turning towards natural medicines. Now-a-days, phytochemical studies on traditional therapeutic plants are acquiring significance. In this aspect a phytochemical screening was carried out on different solvent extracts of the leaves of Pterocarpus santalinus L.f., which is about to get endangered. Research studies on this plant revealed the presence of glycosides, alkaloids, flavonoids, phytosteroids and phenolic compounds. An alkaloid, 5-hydroxy-N,N-dimethyltryptamine was isolated from the methanol (Me) extract by column chromatography. Its structure was confirmed by spectroscopic analysis. Its antimicrobial activity was tested against two pathogenic bacterial strains acquired from contaminated paneer (Indian Cheese) which were identified as Escherichia coli (gram –ve) and Staphylococcus aureus (gram +ve) bacteria. The compound expressed better antimicrobial potential against S.aureus than E.coli, with Maximum Inhibition Zone (MIZ) and Minimum Inhibition Concentration (MIC) values in the range of 1.5-2.0 cm and 0.5-1.0 mg/ml respectively.

Keywords: *Pterocarpus santalinus* L.f., phytochemicals, antimicrobial assay, 5-hydroxy-N,N-dimethyltryptamine (5-HO-N,N- DMT), column chromatography, *Staphylococcus aureus*, *Escherichia coli*, Maximum Inhibition Zone (MIZ) and Minimum Inhibition Concentration (MIC).

Introduction

Pterocarpus santalinus is an Indian conventional medical plant belongs to Leguminosae (Fabaceae) family, commonly known as 'Red Sanders'. It is an endangered plant species whose growth is constrained to tropical nations such as India, Sri Lanka, China etc[1]. It is renowned for its elegant nature and spectacular colour of its wood, usually used in preparation of some royal furniture and special musical instruments. This plant is famous for its therapeutic values in Ayurveda, it cures skin diseases, debility, scorpion bite and ulcers[2]. In preliminary studies, phytochemical analysis was done on different parts of this plant showed the presence various phytochemicals like tannins, phytosteroids, alkaloids, flavonoids, anthocyanins, terpenoids, phenols and glycosides[3]. This plant is also well-known for its antidiabetic[4], anti-inflammatory[5], antiplasmodial[6], antihyperglycemic[7], antifungal[8] and antimicrobial[9] activities. Six terpenoids[10], an acylated isoflavone glucoside[11], savinin and calocedrin[12], two aurone glucosides[13], one phenanthrenedione and one chalcone[14], one coumarin[15] were reported as isolated from this plant. The crude Me extracts of these plant parts were assessed for phytochemicals and antimicrobial activity. The crude extracts of leaves and flowers showed the occurrence of major phytochemicals and good antimicrobial activity against the microbes. On continuation of our studies an alkaloid was isolated from Me extract of leaves.

Materials and methods

Collection: Leaves of *Pterocarpus santalinus* L.f. were procured from Seshachalam hills in Eastern Ghats, Tirupathi, A.P., India. The plant sample authentication was done by Prof B. Ravi Prasad Rao, with voucher no- 48799 has been deposited in Botany Department herbarium at Sri Krishna devaraya University, Ananthapur, A.P., India. The collected leaves of *Pterocarpus santalinus* L.f. plant was shown in Figure-1.

Figure-1: *Pterocarpus santalinus* L.f. leaves.

Extraction: These leaves were cleaned with water, dried in shade, grounded into powder, then extracted with six solvents from polar to non-polar viz, Me, Ethanol (Et), ethyl acetate (Ea), chloroform (Ch), carbon tetrachloride (CC) and n-Hexane (n-He) at room temperature. 500gm of powder was extracted with 1500ml of solvent by vigorous shaking and by soaking it overnight then filtered with Whatmann filter paper. The residue was repeatedly extracted in the same method for four days, until the filtrate becomes pale. The solvents were recycled from the collected filtrates on Heidolph Rota-evaporator under vacuum. The yields of extracts and extraction efficiencies of all solvents were calculated and showed below as Table-1.

Table-1: Yields of crude extracts of *Pterocarpus santalinus* L.f. leaves and extraction efficiencies of all solvents

Solvent	Yield (gms)	Extraction efficiency (%)
Me	210.0	42.0
Et	147.0	29.4
Ea	44.5	8.9
Ch	42.5	8.5
CC	30.0	6.0
n-He	26.0	5.2

Phytochemical screening[16]: 10gm of each crude extract was undergone qualitative phytochemical screening, whose results were tabulated as Table-2.

Isolation: The Me crude extract of leaves was separated by column chromatography, using silica gel of 100-200 mesh with n-He as stationary phase and eluted with six solvents from polar to non-polar viz, Me(100ml), Et(100ml), Ea(100ml), Ch(100ml), CC(100ml), n-He(100ml). Among these six fractions Me fraction showed the highest antimicrobial activity. So, it was again separated using column chromatography technique with silica gel in n-He as stationary phase and eluted with Ch-Me gradient solvent system (5/1, 4/1, 3/1, 2/1, 1/1) resulting in five fractions from F-1 to F-5. These fractions were sent for HPLC purification test, among which F-1 showed highest purity of 82%. F-1 was again subjected to repeated column chromatography and tested for single spot in Thin-layer Chromatography (TLC). Then a pure greenish-yellow coloured powder of 15.3gm weight was isolated from F-1, with melting point at 145.7°C. Its structure was elucidated by LCMS, IR, ^{1}H NMR and ^{13}C NMR spectroscopic analysis.

Table-2: Qualitative phytochemical screening of *Pterocarpus santalinus* Lf., leaf extracts.

Phytochemical	Qualitative test	Solvent extract					
		Me	Et	Ea	Ch	CC	n-He
Alkaloids	Mayers Reagent	+	+	-	-	-	-
	Wagners Reagent	+	+	-	-	-	-
	Hagers Reagent	+	+	-	-	-	-
	Dragendorffs Reagent	+	+	-	-	-	-
Glycosides and carbohydrates	Molishs Test	+	+	-	-	-	-
	Fehlings Test	+	+	-	-	-	-
	Barfoeds Test	+	+	-	-	-	-
	Benedicts Test	+	+	-	-	-	-
	Borntragers Test	+	+	-	-	-	-
	Legals test	+	+	-	-	-	-
Saponins	Foam test	+	+	-	-	-	-
Proteins and Amino Acids	Millons Reagent	+	+	-	-	-	-
	Biurett Reagent	+	+	-	-	-	-
	Ninhdrin Reagent	+	+	-	-	-	-
Phytosteroids	Libermann's-Buchard's Test	+	+	+	+	+	+
Oils & fats	Spot Test	-	-	-	-	-	-
	Saponification Test	-	-	-	-	-	-
Phenolics & Flavonoids	Ferricchloride Test	+	+	-	-	-	-
	Gelatin Test	+	+	-	-	-	-
	Lead acetate Test	+	+	-	-	-	-
	Alkaline Reagent	+	+	-	-	-	-
	Mg & HCl Reduction	+	+	-	-	-	-
Gums & Mucilages	Alcohol- 95% test	-	-	-	-	-	-

Me- Methanol, Et- Ethanol, Ea- Ethyl acetate, Ch- Chloroform, CC- Carbon tetrachloride, n-He = n-Hexane, (+) – positive, (–) – negative.

Application - Antimicrobial susceptibity test[17]: After structural elucidation, remaining compound was completely exhausted for preparing the test solution of 5-hydroxy-N,N-dimethyltryptamine with dimethyl sulphoxide (DMSO) to perform antimicrobial activity test using Kirby-Baurer disk diffusion method[18] against two human pathogenic bacterial strains *Escherichia coli* (gram –ve) and *Staphylococcus aureus* (gram +ve) among which *S.aureus* was identifies as a major contaminant in contaminated paneer[19]. These microorganisms were obtained from environmental sources of contaminated Paneer (Indian cheese) and were identified using 16S rDNA sequencing with the help of Credora Life Sciences, Bangalore. The bacteria was first grown upon nutrient agar (NA) at 37ºC for a period of 24 hrs and 0.6cm diameter wells were made on the infusion agar seeded with bacteria, then the test solution suspended in DMSO was dropped onto each paper disc (100 µl/ml) for all prepared concentrations. The plates were incubated near 37ºC (24 hrs). The antibacterial potential was assessed by measuring the growth inhibition zone diameter, surrounding the disc. Every experiment was done in triplicate, for which statistical analysis was performed and MIZ mean values of these three independent triplicates along with standard deviation were measured.

Procedure: The pure culture plate of the bacterial strains to be tested were selected and a colony was emulsified from the plate in the sterile salt solution aseptically. The saline solution was mixed carefully to make sure that no solid material of colony was seen in the salt solution. A sterile swab was immersed into the broth of organism cultured and squeezed smoothly against inner side of the test tube so as to remove extra liquid in the swab. The swab with the bacteria was used to draw a streak on sterile Nutrient Agar plate for a territory of growth. Later the plates were incubated for 1 hour near 37°C. After completion of the incubation, 0.6cm diametered wells were made and different concentrations (0.5mg/mL, 1mg/ml, 2.5mg/ml, 5 mg/ml) of test solution was added to each well, except for one well which was added with negative control DMSO. The plates were incubated for 24hours around 37°C temperature. Then the diameter of the inhibition zone was determined using a metric ruler. The measurements got from the individual samples were compared with the synthetic standard Ampicillin to determine the zone of sensitivity. The MIZ of 5-HO-N,N-DMT against *Escherichia coli*, *Staphylococcus aureus*, and DMSO in different concentrations with Ampicillin as positive standard after 24 hrs of incubation were showed in Table-4.

Results and discussion

High Performance Liquid Chromatography (HPLC) test: The F-1 fraction's HPLC chromatogram of leaf Me extract showed highest purity of 82% among all five fractions obtained from column chromatography, the result was shown in Figure-2.

Yields and extraction efficiencies: Yields and extraction efficiencies of all the six solvents used in *Pteropcarpus santalinus* L.f. leaf extraction in Table-1 shows that, among six solvents Me gave highest yield of 210gms showing highest extraction efficiency of 42%.

Phytochemical screening: In phytochemical analysis, phytochemicals present in all solvent extracts tabulated in table-2 displays that Me and Et extracts showed the presence of all phytochemical metabolites except fixed oils, fats, gums and mucilages. As Me crude extract had highest yield and revealed the presence of more number of phytochemicals, it was selected for further studies.

Spectral Interpretation: The data of all spectrums was collected, analysed and interpreted as followed in order to identify the compound isolated.

The molecular weight 204.07g.mol^{-1} of this compound was confirmed by the Liquid Chromatography Mass spectrum (LCMS) of Shimadzu LCMS - 2010A with [M^++1] peak at m/z 205.07, [M^+- 44] peak at m/z 160, [M^+-58] peak at m/z 146 and [M^+-72] peak at m/z 132. The fragmentation peaks at m/z- 160, 146, 132 indicates dimethyl amine, trimethyl amine and dimethyl ethyl amine ion fragments respectively which were shown in Figure-3.

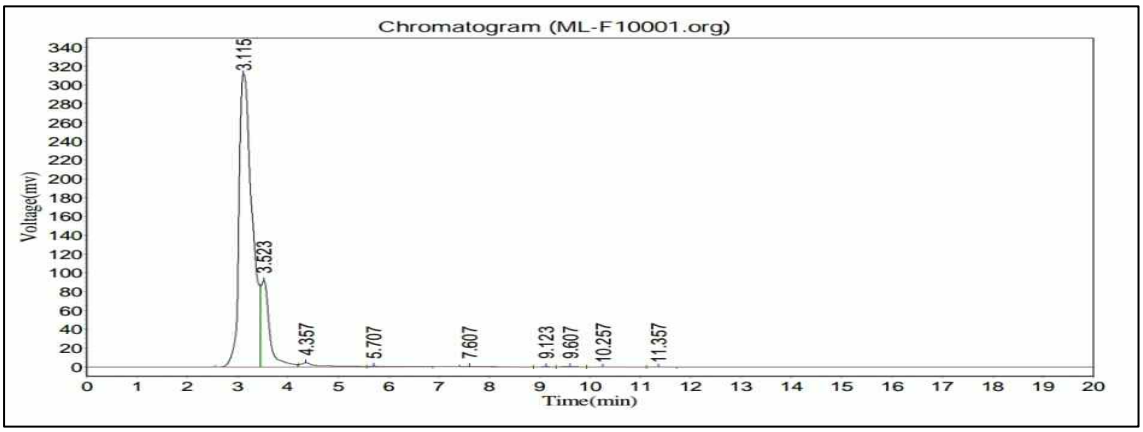

Figure-2: HPLC chromatogram of F-1 fraction of Leaf Me extract.

The Infra Red (IR) spectrum in Figure-4, shows the functional group bands of hydroxyl group at 3316cm^{-1}, a methyl -CH stretching at 2951cm^{-1}, a 1º amine –(C-N)- stretching at 1637cm^{-1}, an aromatic amine =(C-N)- stretching at 1350cm^{-1}, an aromatic –C=C- stretching at 1515cm^{-1}, a phenol =C-O- stretching at 1208cm^{-1}, an aliphatic amine –CH stretching at 1041cm^{-1} and m-disubstituted benzene at 993cm^{-1}.

The ^{1}H Nuclear Magnetic Resonance (NMR) spectrum was recorded at 400 MHz ($CDCl_3$) revealed a six proton sharp singlet (δ_H 2.26, s) of methyl groups at C-4' & C-5' carbons, two methylene protons (δ_H 2.55, d, J=7.1; δ_H 2.63, d, J=7.1) of C-1' & C-2' carbons in methylene group attached to the indole ring in the upfield, one aromatic hydroxyl proton broad singlet (δ_H 5.35) of –OH group attached to indole ring at C-5 carbon, one aromatic proton triplet of C-6 carbon (δ_H 6.76 t, J=7.5, J=1.5), two aromatic proton doublets of C-4 & C-7 carbons (δ_H 7.05, d, J=1.5, δ_H 7.15, d, J=7.5), one aromatic proton sharp singlet of C-2 carbon (δ_H 7.47) and one aromatic amine proton broad singlet of N-1 (δ_H 10.1, s) of indole ring in the downfield. All the above data of ^{1}H NMR spectrum was displayed in Figure-5.

In ^{13}C-NMR spectrum which was recorded at 100 MHz ($CDCl_3$) 11 carbon signals were observed, which includes one sp^3-C peak of two methyl groups(C-4', C-5') at δ_C 47.0, two $-CH_2-CH_2-$ carbon peaks (δ_C 22.6, δ_C 64.2) of C-1' & C-2' carbons in upfield and eight aromatic carbons of indole ring in the downfield ranging from δ_C 110 to δ_C 160. Aliphatic amine group carbon found to be attached to C-2' carbon gave peak at δ_C 64.2 and C-5 carbon found to be attached to a hydroxyl group gave a peak at δ_C 152.4. The Figure-6 given below shows the ^{13}C NMR spectrum data completely.

The entire data of ^{1}H-NMR spectrum and ^{13}C-NMR spectrum was tabulated in Table-3.

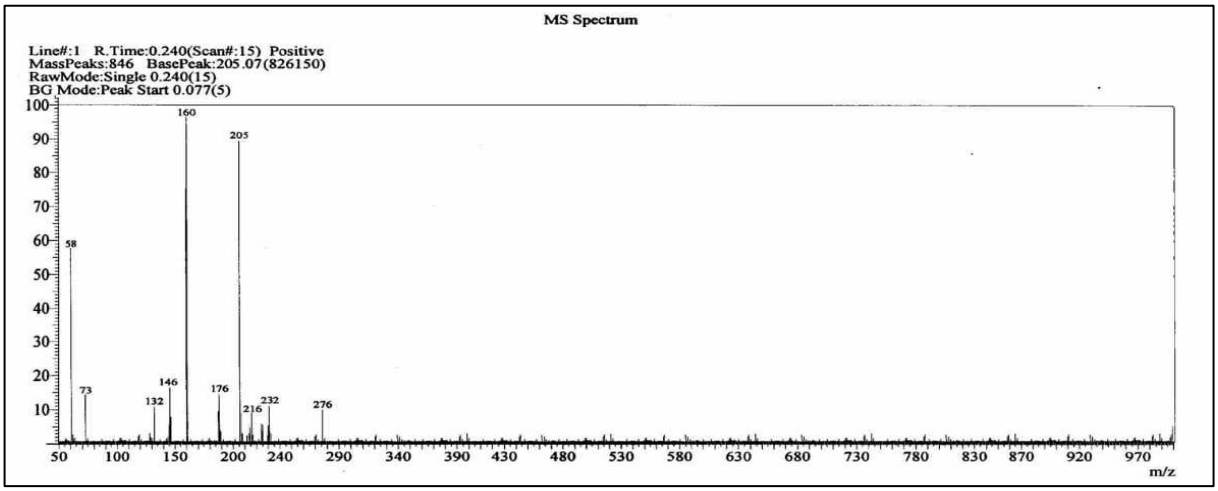

Figure-3: LCMS spectrum of 5-hydroxy-N-N-dimethyltryptamine.

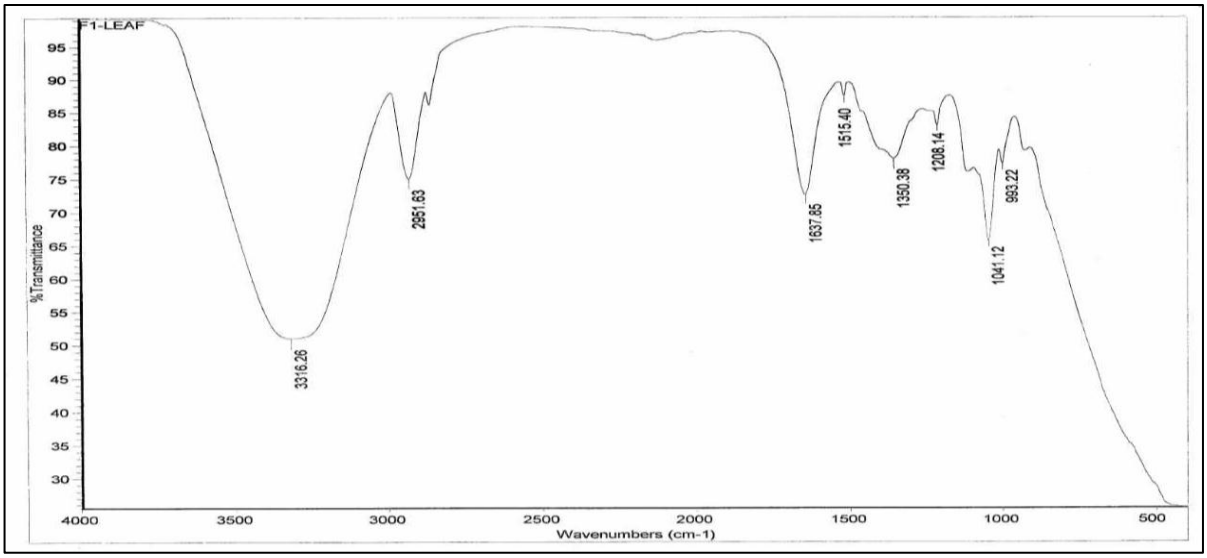

Figure-4: IR spectrum of 5-hydroxy-N-N-dimethyltryptamine.

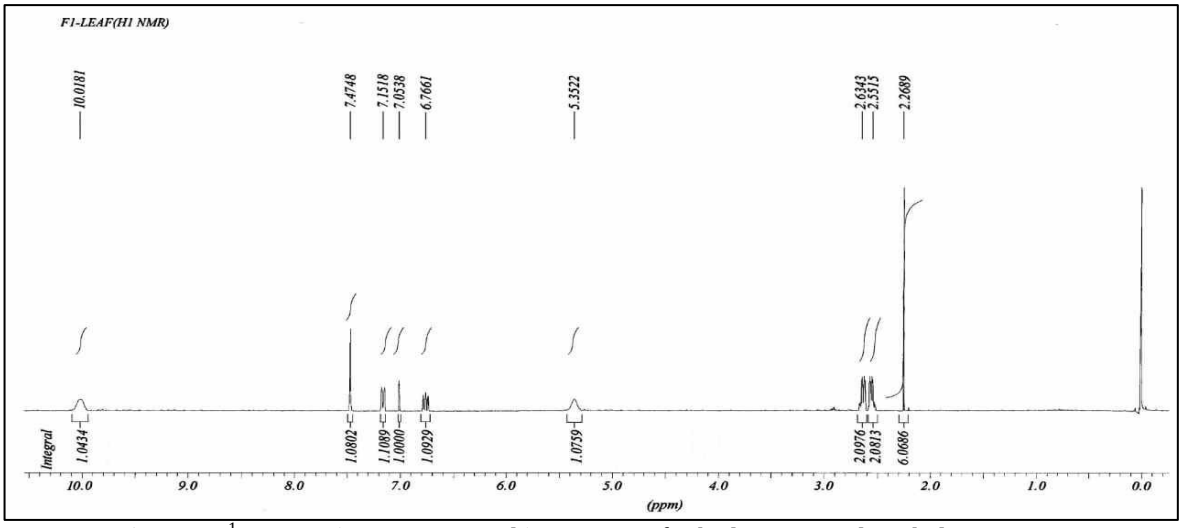

Figure-5: ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of 5-hydroxy-N-N-dimethyltryptamine.

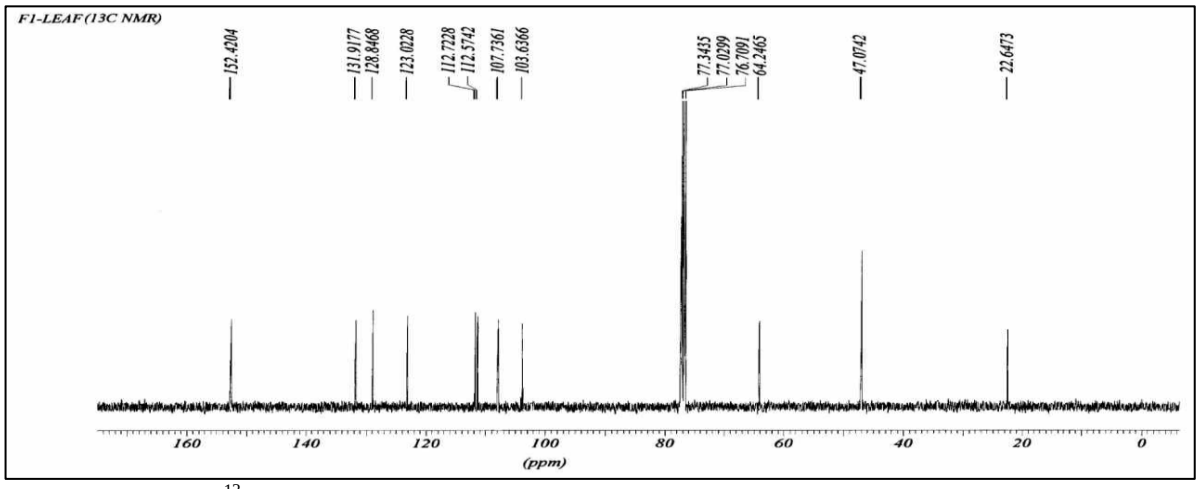

Figure-6: ^{13}C-NMR at 100 MHz ($CDCl_3$) spectrum of 5-hydroxy-N-N-dimethyltryptamine.

Table-3: ^{1}H-NMR and ^{13}C-NMR data of 5-hydroxy-N-N-dimethyltryptamine.

Atom position	No. of Protons	^{1}H *(δ_H)	^{13}C *(δ_C)
N-1	1	10.1 *s	-
C-2	1	7.47 s	123.0
C-3	0	-	107.7
C-4a	0	-	128.8
C-4	1	7.05 *d (1.5)	103.6
C-5	0	-	152.4
C-5-OH	1	5.35 s	-
C-6	1	6.76 *t (7.5) (1.5)	112.7
C-7	1	7.15 d (7.5)	112.5
C-7a	0	-	131.9
C-1'	2	2.55 d (7.1)	22.6
C-2'	2	2.63 d (7.1)	64.2
N-3'	0	-	-
C-4'	3	2.26 s	47.0
C-5'	3	2.26 s	47.0

*(δ_H) – proton chemical shift, (δ_C) – carbon chemical shift, s = singlet, d = doublet, t = triplet.

Table-4: MIZ of 5-hydroxy-N,N-dimethyl tryptamine *(5-HO-N,N-DMT) antimicrobial assay.

Test Sample	*Conc. mg/ml	Maximum Inhibition Zone (MIZ) in cms							
		E. coli				*S. aureus*			
		I	II	III	Mean ± Std Dev	I	II	III	Mean ± Std Dev
5-HO-N,N-DMT	5.0	0.19	0.15	0.21	0.18 ± 0.03	1.42	1.52	1.62	1.52 ± 0.10
5-HO-N,N-DMT	2.5	0.22	0.23	0.26	0.24 ± 0.02	2.01	2.04	2.09	2.05 ± 0.04
5-HO-N,N-DMT	1.0	0.45	0.41	0.39	0.42 ± 0.03	2.53	2.47	2.63	2.54 ± 0.08
5-HO-N,N-DMT	0.5	0.81	0.79	0.77	0.79 ± 0.02	3.12	3.33	3.06	3.17 ± 0.14
*Amp	0.5	1.10	1.02	1.07	1.06 ± 0.04	2.59	2.56	2.5	2.55 ± 0.05
*DMSO	*Neg. control	0.00	0.00	0.00	0.00 ± 0.00	0.00	0.00	0.00	0.00 ± 0.00

*5-HO-N,N-DMT = 5-hydroxy-N,N-dimethyltryptamine, DMSO - Dimethyl Sulphoxide, Amp - Ampicillin (positive standard), Conc - concentration of test solution, Neg – Negative

Antimicrobial susceptibity test: The incubation results in Table-4 showed that the isolated compound has good antimicrobial activity against *S.aureus* bacterial strain in all concentrations than *E.coli* bacterial strain, but it was found to be less when compared with the standard Ampicillin. The MIZ and MIC values of the sample against the microbes were in the range of 2.0–1.5cm, 0.5 – 1mg/ml respectively.

Conclusion

From all the results and by correlating all the above data the isolated compound with molecular formula $C_{12}H_{16}N_2O$ and molecular weight of 204.07g.mol^{-1} was concluded as 5-hydroxy-N,N-dimethyl tryptamine, an alkaloid commonly called as Bufotenine. Its melting point was recorded at 145.7°C. The compound identity was also confirmed by comparing the spectroscopic details of the compound with the reported literature values[20,21] which were in fine coincidence and its structure was shown in Figure-7.

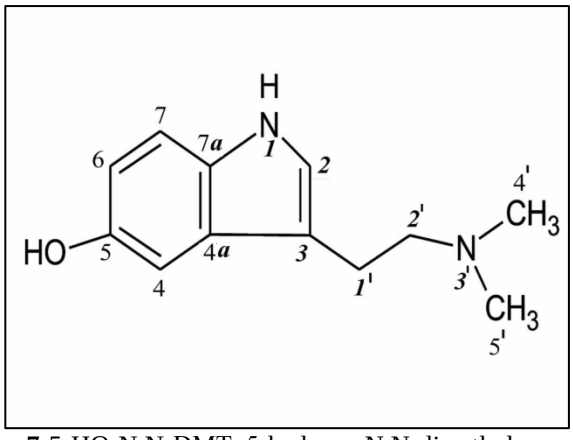

Figure-7:5-HO-N,N-DMT–5-hydroxy-N,N-dimethyl-tryptamine.

The antimicrobial assay revealed that this compound has effective antimicrobial activity against human pathogenic bacterial strains. 5-hydroxy-N,N-dimethyl tryptamine is a psychoactive drug[22]. It is a Schedule I drug in US[23] and Class A drug in UK[24].

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