Chapter-4

Results

4.1 Fungal material collection

S. stipitatum was collected from Malabar areas of Kerala (Thrissur, Palakkad, Malappuram, Kozhikode, Kannur, Kasargod, Wayanad). Mainly from the tribal colonies and forests of Palakkad (Plate 1). Its identity is confirmed at National Fungal Culture Collection of India (NFCCI) and the specimen is deposited at Ajrekar Mycological Herbarium (AMH) with accession number: AMH-10322.

4.2 Molecular characterization

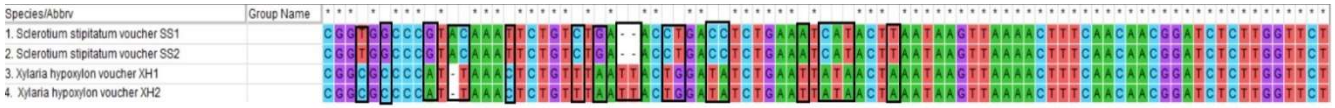
DNA barcode analysis using ITS barcode region was carried out to confirm the species identity of the freshly collected fungal specimens. ITS barcode sequences generated were submitted to GenBank and the following accession numbers were obtained; *S. stipitatum* (384 bp) (MZ330799- MZ330800) and *Xylaria hypoxylon* (363 bp) (MZ330801-MZ330802). BLAST similarity search showed more than 95.32% homology with ITS sequence of *Xylaria acuminatilongissima* available at the GenBank (Figure 2). Further, multiple sequence alignment of ITS barcode sequences obtained in this study from *S. stipitatum* and *X. hypoxylon* revealed several nucleotide changes. (Figure 3). Dendrogram based on ITS DNA barcode sequences of both genera also displayed two distinct clades (Figure 4).

Plate 1. *S. stipitatum* in various sizes and shapes collected from natural habit

Figure 2. BLAST similarity search results between *S. stipitatum* and *X. acuminatilongissima*

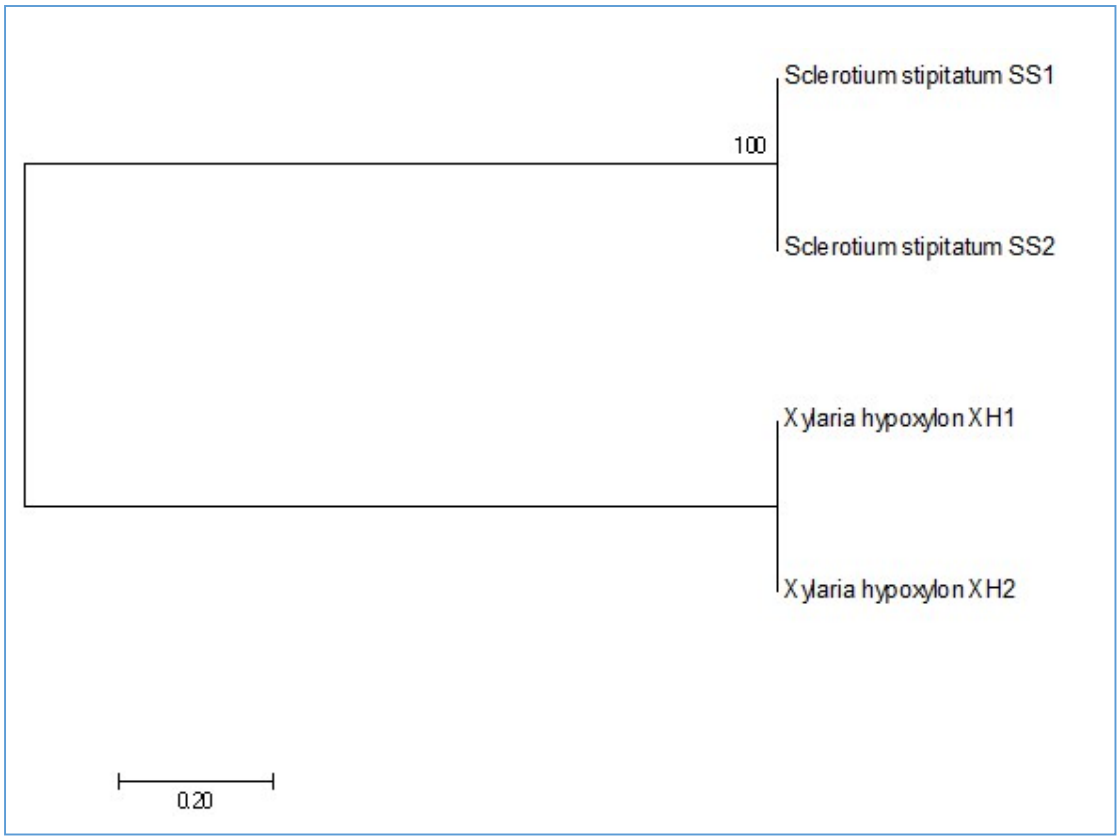
Xylaria acuminatilongissima voucher K(M) 191670 18S ribosomal RNA gene, partial sequence; internal transcribed spacer ...	NA	438	498	80%	1e-118	95.32%	506	KP067787.1
Xylaria acuminatilongissima BCRC 34211 ITS region; from TYPE material	NA	438	506	84%	1e-118	95.32%	561	NR_147516.1
Xylaria sp. SCJH internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed space...	NA	433	489	79%	6e-117	94.95%	486	HM469970.1
Xylaria sp. 3 HMH-2010c 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA g...	NA	433	502	81%	6e-117	94.95%	559	GU324756.1
Xylariaceae sp. OTU5 isolate 518.F8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete ...	NA	433	493	80%	6e-117	94.96%	491	FJ425673.1
Xylaria nigripes isolate 653 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA ...	NA	414	478	80%	2e-111	95.42%	581	GU324755.1
Xylaria nigripes 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and j...	NA	414	478	80%	2e-111	95.42%	581	EU179868.1
Xylaria sp. AMS4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, an...	NA	407	407	67%	4e-109	95.02%	500	EU164407.1
Xylariaceae sp. OTU4 isolate 335 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete seq...	NA	405	405	68%	1e-108	94.34%	470	FJ425672.1
Kretzschmaria pavimentosa isolate MY94 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	NA	403	403	69%	5e-108	94.07%	599	MN613119.1
Kretzschmaria pavimentosa isolate MY92 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	NA	403	403	69%	5e-108	94.07%	596	MN613118.1
Kretzschmaria pavimentosa isolate MY91 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer...	NA	403	403	69%	5e-108	94.07%	598	MN613117.1

Figure 3. Result of multiple sequence alignment of ITS barcode sequences of *S. stipitatum* and *X. hypoxylon*



Species/Abbrv	Group Name
1. Sclerotium stipitatum voucher SS1	
2. Sclerotium stipitatum voucher SS2	
3. Xylaria hypoxylon voucher XH1	
4. Xylaria hypoxylon voucher XH2	

Figure 4. Dendrogram based on ITS DNA barcode sequences of *S. stipitatum* and *X. hypoxylon*



4.3 Preparation of fungal extracts

About 20 grams of powder was sequentially extracted with solvents like petroleum ether, chloroform, acetone, ethanol and water using soxhlet apparatus. Distillation over a water bath was used to remove the solvents. Then the extracts were stored in glass bottles and kept in refrigerator for further studies.

4.3.1 Chemical analysis

For the chemical screening the extracts prepared using soxhlet apparatus was used after evaporating the solvents completely. Petroleum ether extract consist of phytosterol only. Chloroform extract contain triterpenoids and glycosides in addition with phytosterol. Acetone extract contains lactones and tannins. Ethanol extract consists of alkaloids, flavonoids, phenols, aleurone grains and saponins. Distilled water extract has phenols and naphthoquinones (Table 2).

Table 2. Chemical analysis of *S. stipitatum*

Chemical constituents	Test	Petroleum ether	Chloroform	Acetone	Ethanol	Dis. water
Phytosterol	Salkowski test	+	+	-	-	-
Triterpenoids	Liebermann-Burchard test	-	+	-	-	-
Saponins	Foam test	-	-	-	+	-
Alkaloids	Mayer's test	-	-	-	+	-
	Wagner's test	-	-	-	+	-
Flavonoids	Lead acetate test	-	-	-	+	-
	Alkaline test	-	-	-	-	-
Lactones	Legal's test	-	-	+	-	-
Tannins	Lead acetate test	-	-	+	-	-
	Braemer's test	-	-	-	-	-
	Catechin test	-	-	-	-	-
Sterols	Liebermann-Burchard test	-	-	-	-	-
Resins	General test	-	-	-	-	-
Proteins	Ninhydrin test	-	-	-	-	-
	Biuret test	-	-	-	-	-
Glycosides	Keller-Kiliani test	-	+	-	-	-
Coumarins	General test	-	-	-	-	-
Monosaccharides	Molisch's test	-	-	-	-	-
	Benedict's test	-	-	-	-	-
	Barfoed's test	-	-	-	-	-
	Seliwanoff's test	-	-	-	-	-
Polysaccharides	Iodine test	-	-	-	-	-
	Molisch's test	-	-	-	-	-
	Seliwanoff' test	-	-	-	-	-
Volatile oils	General test	-	-	-	-	-
Phenol	General test	-	-	-	+	+
Naphthoquinones	Juglone test	-	-	-	-	+
Aleurone grains	General test	-	-	-	+	-

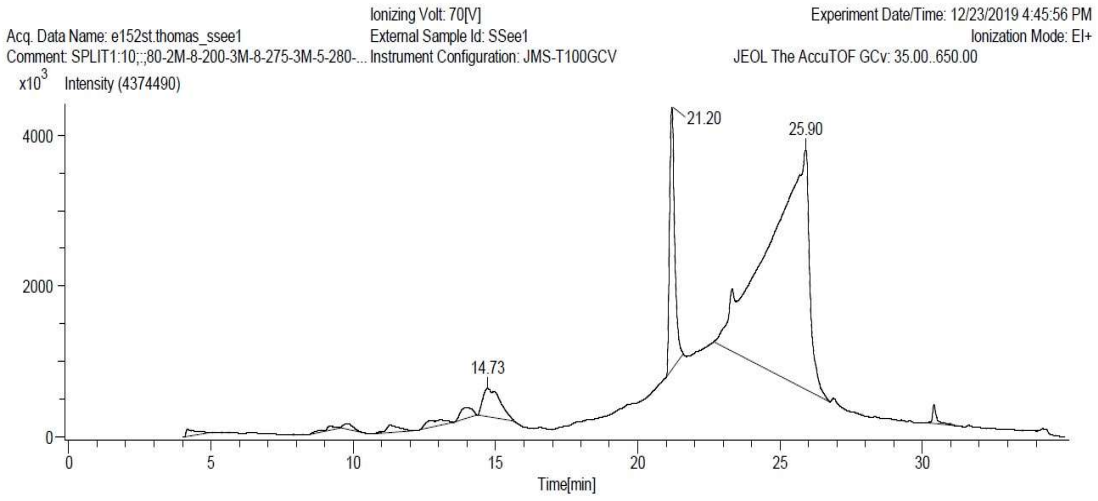
Since most number of compounds are present in ethanol extract it is chosen for the further studies.

4.3.2 GC-MS Analysis

The ethanol extract of *S. stipitatum* has the following composition: 2-Propanone, 1-(dimethylamino)-; 2-Pyrrolidinone; N-Trimethylsilyl-2-pyrrolidinone; 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester; Hexanediamide, N,N'-di-benzoyloxy-; 2,5-Methylene-d,l-rhamnitol; 2,5-Methylene-d,l-rhamnitol; 2,5-Methylene-d,l-rhamnitol; Ribitol; 1,2-Benzenecarboxylic acid, diisooctyl ester. The retention time of above compounds were 4.16, 9.18, 9.80, 11.30, 13.05,13.97, 14.73, 21.20, 25.90, and 30.41 respectively.

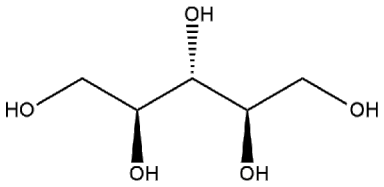
The compounds ribitol and 2,5-Methylene-d, l-rhamnitol are the most abundant compounds present in the extract. All other compounds are present in only a minute quantity compared to this. The structure of compounds are also elucidated. (Figure 5 and 6).

Figure 5. GC-MS spectrum of ethanol extract of *S. stipitatum*

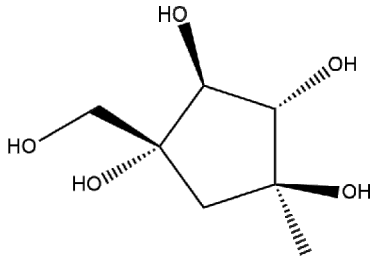


Peak Number	Time [min]	Type	Peak Width(FWH [min]	Area [Intens. * sec]	Height	Description	Start Point		End Point	
							Time[min]	Height	Time[min]	Height
1	4.16	BB	0.9141	2343435.76	93027.72		4.08	348	5.03	58452
2	9.18	BB	0.4188	1349041.27	53854.07		8.50	36202	9.52	118512
3	9.80	BB	0.3969	1779566.47	73279.37		9.54	120015	10.44	49870
4	11.30	BB	0.5489	3150143.03	97710.57		10.75	43413	12.13	82538
5	13.05	BB	1.0027	3868192.87	72597.01		12.34	93584	13.57	195494
6	13.97	BB	0.5241	4280565.76	149085.98		13.57	195494	14.35	289733
7	14.73	BB	0.6711	16019591.27	377000.41		14.37	288970	15.65	212437
8	21.20	BB	0.1915	43971058.94	3477945.37		21.00	789691	21.60	1103353
9	25.90	BB	1.8572	311300866.46	3178619.18		22.67	1264878	26.77	461983
10	30.41	BB	0.1109	2690236.09	259402.16		30.10	188196	31.36	140512

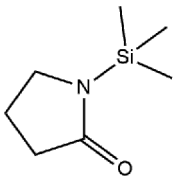
Figure 6. Structure of compounds obtained by GC-MS analysis of ethanol extract of *S. stipitatum*



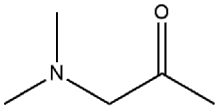
Ribitol



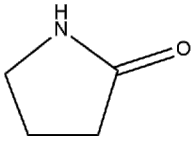
2,5-Methylene-dl-rhamnitol



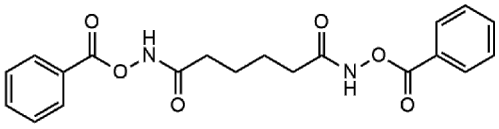
N-Trimethylsilyl-2-pyrrolidinone



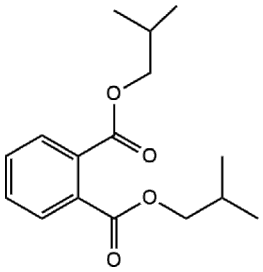
2-Propanone,1-(dimethylamino)-

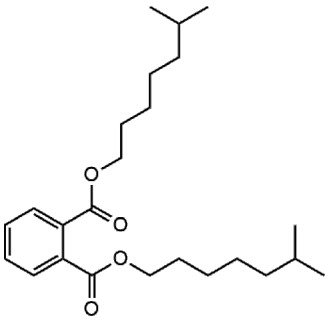


2-Pyrrolidinone



Hexanediamide,N,N'-di-benzoyloxy-





1,2-Benzenedicarboxylic acid, diisooctyl ester

1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester

4.3.3 LC-MS ANALYSIS

LC-MS performed in dual ion mode showed the presence of many bio-active compounds. The most abundant compounds present in the extract are amino acid combination Arg Phe Arg, eflornithine, monobenzone, C16 sphinganine.

Figure 7. Chromatogram of LC-MS analysis in +ve ESI mode of ethanol extract of *S. stipitatum*

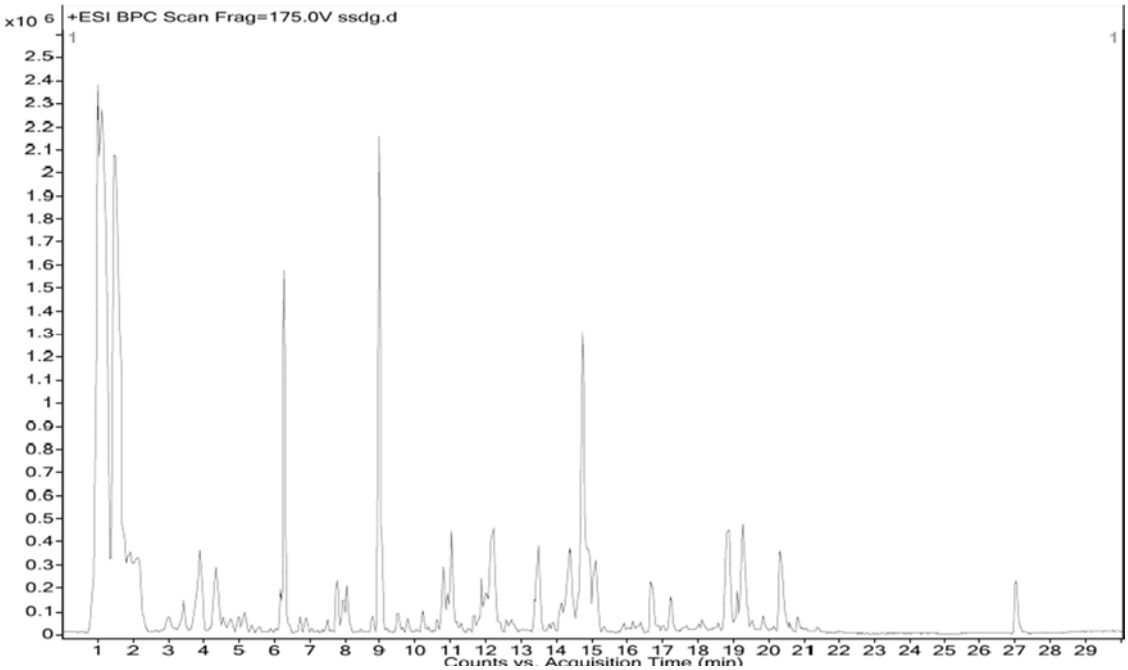


Figure 8. Chromatogram of LC-MS analysis in -ve ESI mode of ethanol extract of *S. stipitatum*

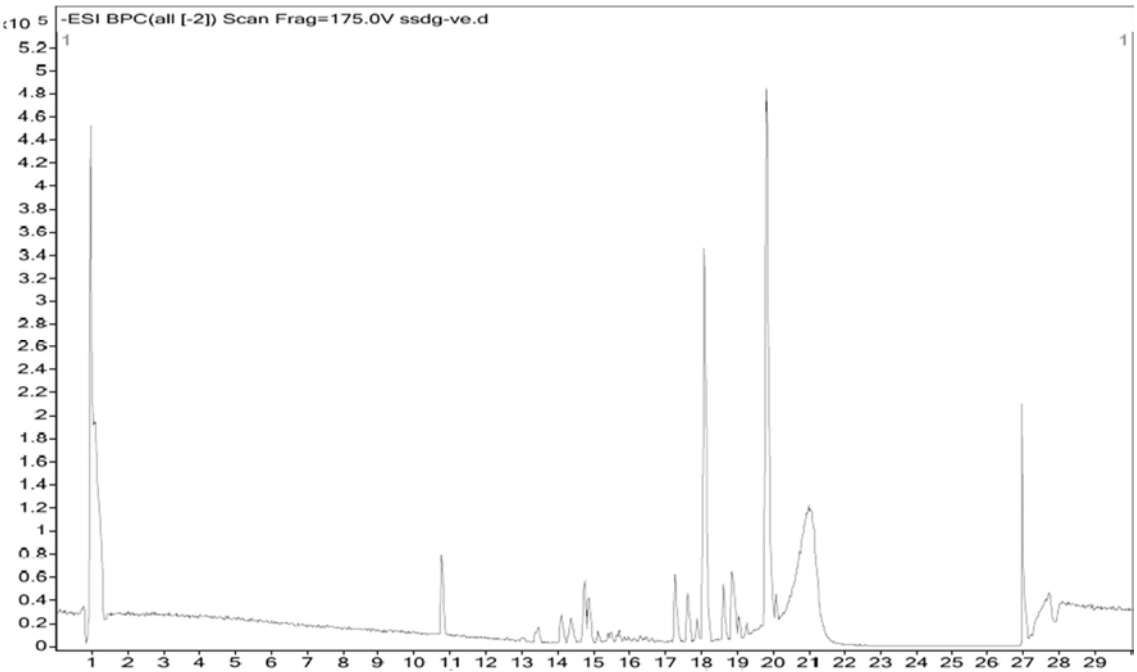


Table 3. List of compounds obtained through LC-MS analysis (+ve ESI mode) of ethanol extract of *S. stipitatum*

RT	Mass	Abundance	Name	Formula
0.97	-	159553	-	-
1.004	-	-	-	-
1.008	596.3082	-	(5b,7a,12a)-(1,3-dihydro-5- nitro-1,3-dioxo-2H-isoindol-2- yl)methyl ester	C_{33} H_{44} N_2 O_8
1.023	204.0454	-	PYROGALLIN	C_{11} H_8 O_4
1.029	182.0751	53097	9-Hydroxyfluorene	C_{13} H_{10} O
1.093	216.0714	176668	Diphenylmethylphosphine oxide	C_{13} H_{13} O P
1.128	358.1348	-	Deacetyl-N- monodemethyldiltiazem	C_{19} H_{22} N_2 O_3 S
1.306	412.1406	-	Gln Cys Tyr	C_{17} H_{24} N_4 O_6 S
1.416	200.082	-	Monobenzone	C_{13} H_{12} O_2
3.427	596.3082	21575	(5b,7a,12a)-(1,3-dihydro-5- nitro-1,3-dioxo-2H-isoindol-2- yl)methyl ester	C_{33} H_{44} N_2 O_8
5.158	537.3372	29641	GPEtn(10:0/11:0)[U]	C_{26} H_{52} N O_8 P
6.541	-	15327	-	-
7.953	297.1847	38778	Mono-N- desisopropyldisopyramide	C_{18} H_{23} N_3 O
8.083	470.0297	20610	1-Phosphatidyl-1D-myo- inositol 3-phosphate	C_{11} H_{20} O_{16} P_2
8.349	-	49664	-	-
8.362	263.1697	13391	Desmethylmaprotiline	C_{19} H_{21} N
8.434	273.1318	22537	Thr Gly Pro	C_{11} H_{19} N_3 O5
8.945	567.2859	13714	Dihydrodeoxystreptomycin	C_{21} H_{41} N_7 O_{11}
9.796	317.2762	26988	N-methyl arachidonoyl amine	C_{21} H_{35} N O
9.812	273.2715	198447	C16 Sphinganine	C_{16} H_{35} N O_2
10.241	287.2787	31847	C17 Sphinganine	C_{17} H_{37} N O_2
10.977	360.3019	21604	5beta-Cholan-24-oic Acid	C_{24} H_{40} O_2
11.802	-	44774	-	-
11.891	440.212	-	His Arg Glu	C_{17} H_{28} N_8 O_6
11.931	466.1912	111532	Clobetasol propionate	C_{25} H_{32} Cl F O_5

11.986	446.307	57117	(23R)-1alpha,23,25-trihydroxy- 24-oxovitamin D3 / (23R)- 1alpha,23,25-trihydroxy-24- oxocholecalciferol	C_{27} H_{42} O_5
13.054	444.2901	47654	(23R,25R)-1alpha,25-dihydroxyvitamin D3 26,23- lactone / (23R,25R)-1alpha,25-dihydroxycholecalciferol	C_{27} H_{40} O_5
13.447	484.2421	170395	Tyr Phe Arg	C_{24} H_{32} N_6 O_5
13.45	506.2211	65273	1alpha,25-dihydroxy- 26,26,26,27,27,27-hexafluoro- 16,17,23,23,24,24-hexadehydro-19-norvitamin D3 / 1a	C_{26} H_{32} F_6 O_3
13.664	-	64440	-	-
13.797	444.2907	134540	(23R,25R)-1alpha,25-dihydroxyvitamin D3 26,23- lactone / (23R,25R)-1alpha,25-dihydroxycholecalciferol	C_{27} H_{40} O_5
14.25	446.3068	49893	(23R)-1alpha,23,25-trihydroxy- 24-oxovitamin D3 / (23R)- 1alpha,23,25-trihydroxy-24- oxocholecalciferol	C_{27} H_{42} O_5
14.359	471.3136	63018	Terfenadine	C_{32} H_{41} N O_2
14.641	290.2285	-	Androstenediol	C_{19} H_{30} O_2
14.643	424.2499	-	3'alpha-Isopravastatin	C_{23} H_{36} O_7
14.735	918.5854	-	Megalomicin B	C_{46} H_{82} N_2 O_{16}
14.85	-	119709	-	-
14.854	-	59744	-	-
14.947	424.2571	13581	1-heptadecanoyl-sn-glycerol 3- phosphate	C_{20} H_{41} O_7 P
15.04	444.2864	141430	(23R,25R)-1alpha,25-dihydroxyvitamin D3 26,23- lactone / (23R,25R)-1alpha,25-dihydroxycholecalciferol	C_{27} H_{40} O_5
15.074	296.157	27808	Panaxydol chlorohydrine	C_{17} H_{25} Cl O_2
15.149	428.2834	136994	2-glyceryl-PGF2alpha	C_{23} H_{40} O_7
15.191	868.5457	146283	Tilmicosin	C_{46} H_{80} N_2 O_{13}
15.542	477.2894	314627	Arg Phe Arg	C_{21} H_{35} N_9 O_4
15.565	454.3086	87764	Coenzyme Q4	C_{29} H_{42} O_4
15.804	-	11652	-	-
16.08	460.3223	31793	1alpha,24,25,28- tetrahydroxyvitamin D2 / 1alpha,24,25,28-tetrahydroxyergocalciferol	C_{28} H_{44} O_5

16.144	-	22035	-	-
16.666	524.2789	30792	26,26,26,27,27,27-hexafluoro- 1alpha,24-dihydroxyvitamin D3 / 26,26,26,27,27,27-hexafluoro-1alpha,24-	C_{27} H_{38} F_6 O_3
16.73	-	32006	-	-
16.734	480.3152	36463	Crustecdysone	C_{27} H_{44} O_7
16.937	436.3134	15875	4,4-difluoro-1alpha- hydroxyvitamin D3 / 4,4- difluoro-1alpha-hydroxycholecalciferol	C_{27} H_{42} F_2 O_2
19.543	435.0549	22058	Aztreonam	C_{13} H_{17} N_5 O_8 S_2
20.044	-	38844	-	-
20.407	408.2913	29914	3alpha,6beta,7beta-Trihydroxy 5beta-cholan-24-oic Acid	C_{24} H_{40} O_5
20.995	719.2078	11193	Epirubicin glucuronide	C_{33} H_{37} N O_{17}
26.959	387.2497	36872	Lys Gln Leu	C_{17} H_{33} N_5 O_5
26.968	410.216	33117	Tyr Lys Thr	C_{19} H_{30} N_4 O_6
26.973	436.2319	27076	Flurandrenolide	C_{24} H_{33} F O_6
27.015	300.1807	10793	Lys Pro Gly	C_{13} H_{24} N_4 O_4

Table 4. Compounds obtained through LC-MS analysis (-ve ESI mode) of ethanol extract of *S. stipitatum*

RT	**Mass**	**Abundance**	**Name**	**Formula**
1.017	182.0865	200010	Eflornithine	C_6 H_{12} F_2 N_2 O_2
1.035	200.0865	202578	Monobenzone	C_{13} H_{12} O_2
17.246	360.2276	22457	Pregn-4-ene-3,20-dione, 6b,17-dihydroxy-6-methyl-	C_{22} H_{32} O_4
18.641	388.271	23892	o-Hydroxyfinasteride	C_{23} H_{36} N_2 O_3
18.897	334.2491	39301	Tetrahydrodeoxycorticosterone	C_{21} H_{34} O_3
18.906	378.3089	14517	5beta-Cholane- 3alpha,7alpha,12alpha-triol	C_{24} H_{42} O_3
19.789	760.5971	17369	GPA(18:0/22:0)[U]	C_{43} H_{85} O_8 P
19.847	416.2905	45806	(24R,25S)-25,26-epoxy-1alpha,24-dihydroxy-27- norvitamin D3 / (24R,25S)- 25,26-epoxy-1alpha,24- dihydro	C_{26} H_{40} O_4
19.947	424.3074	19103	2alpha-Fluoro-19-nor-22-oxa- 1alpha,25-dihydroxyvitamin D3	C_{25} H_{41} F O_4
20.047	380.3021	43435	24-Nor-5beta-cholane- 3alpha,7beta,22,23-tetrol	C_{23} H_{40} O_4

4.4 In-vitro cytotoxicity study

The in-vitro cytotoxicity assay was conducted in the ethanol extract of *S. stipitatum* using DLA (Dalton's Lymphoma Ascites) and EAC (Ehrlich Ascites Carcinoma) cells. It is determined by the trypan blue dye exclusion method. Percentage cytotoxicity is different in 2 cell types. In both cell lines cytotoxicity gradually increases as the concentration of drug is increased but the rate is more in the case of DLA cell lines. IC_{50} value of cytotoxicity in DLA cell is 191.09 µg/mL. The result of cytotoxicity is given in the tables below (Table 5 and 6).

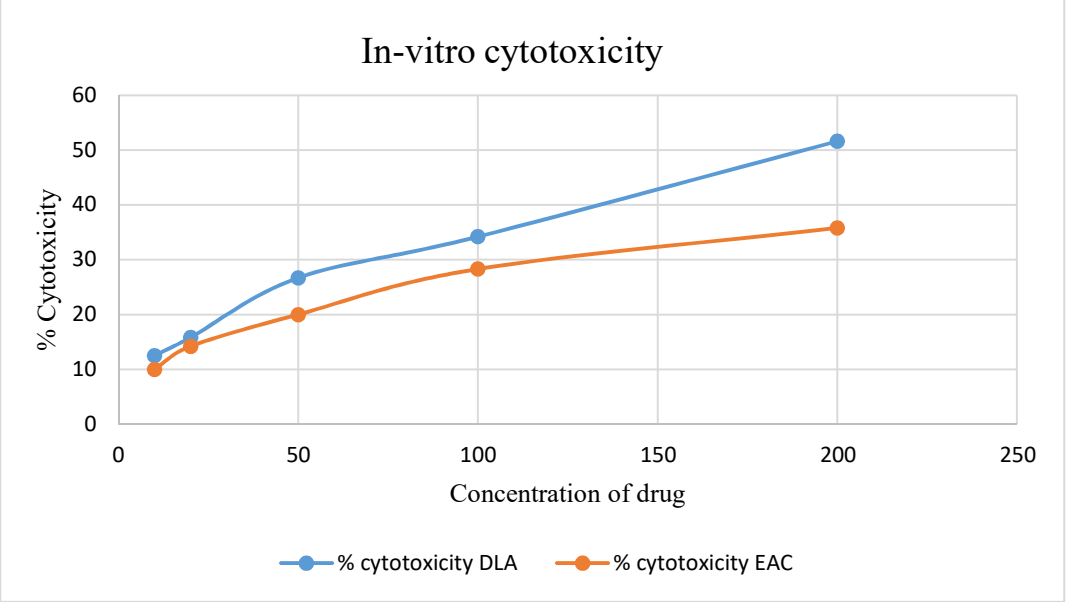
Table 5. Percentage of cytotoxicity of ethanol extract of *S. stipitatum* using DLA cells

Concentration of drug (µg/mL)	**Percentage Cytotoxicity (%)**
10	12.5
20	15.8
50	26.7
100	34.2
200	51.6

Table 6. Percentage of cytotoxicity of ethanol of *S. stipitatum* extract using EAC cells

Concentration of drug (µg/mL)	**Percentage Cytotoxicity (%)**
10	10.0
20	14.2
50	20.0
100	28.3
200	35.8

Figure 9. Graphical representation of percentage cytotoxicity of DLA and EAC cells



4.5 Animal experiments

All the animal experiments were carried out with prior approval from Institutional Animal Ethical Committee with approval number- ACRC/IAEC/18(2) P-2. Following the internationally accepted laboratory animal use and care guidelines and rules of CPCSEA (Approval no. of institution – 149/PO/Rc/S/99/ CPCSEA).

4.5.1 Toxicity study

The acute toxicity study revealed that the administration of ethanol extract was safe up to 2 gram/kilogram body weight (gm/kg b. wt.) with no toxicity or mortality.

4.5.2 In vivo antitumor study

The in vivo antitumor activity was evaluated in solid tumor model and fluid (ascites) tumor model. DLA cells were used in the former one and EAC cells in the latter.

4.5.2.1 DLA-induced solid tumor model

The formation of solid tumor in Swiss albino mice was induced by injecting DLA cell lines intramuscularly into the animals' right-hind limbs. After 24 hrs of

tumor implantation, the extracts and standard drug were given. The ethanolic extracts were given orally for ten days while cyclophosphamide, a conventional medication, was administered intraperitoneally. The tumor development was observed regularly, and the volume of the tumor was measured using a vernier caliper every three days for thirty days. Then the percent inhibition was calculated and shown in table 7.

Table 7. Percentage inhibition of ethanol extract of *S. stipitatum* on DLA induced solid tumor in mice

Groups	**Tumor Volume (cm3) on 5th week (±SE)**	**Percentage inhibition (%)**
Control	3.174 ±0.113	-
Cyclophosphamide (10 mg/kg)	0.738 ±0.077****	76.74
Extract (200 mg/kg)	0.941 ±0.121****	70.35
Extract (50 mg/kg)	1.389 ±0.09****	56.23

Values are expressed as Mean ± SE, n = 6, **** p< 0.0001 compared to control considered as significant.

Figure 10. Antitumor activity of ethanol extract of *S. stipitatum* on solid tumor in mice

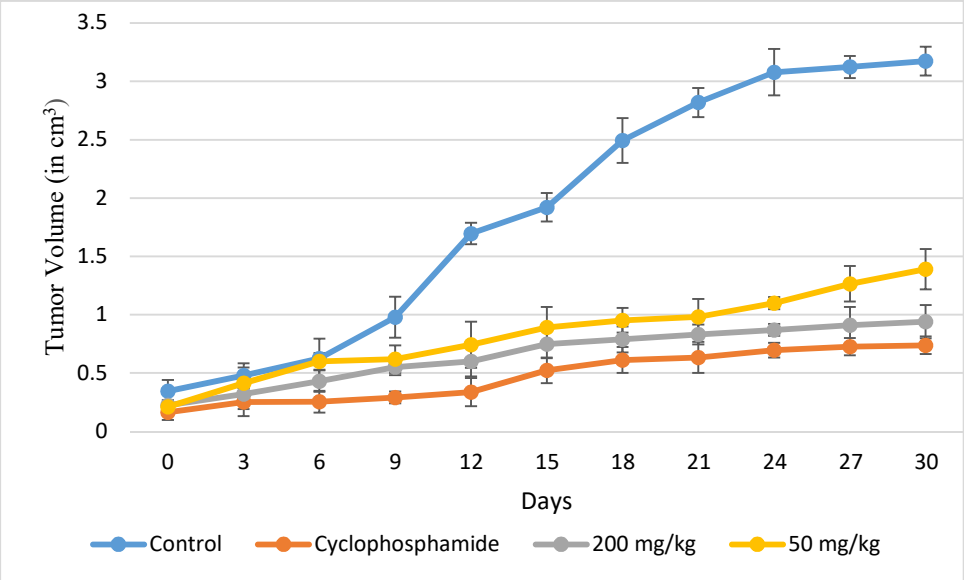
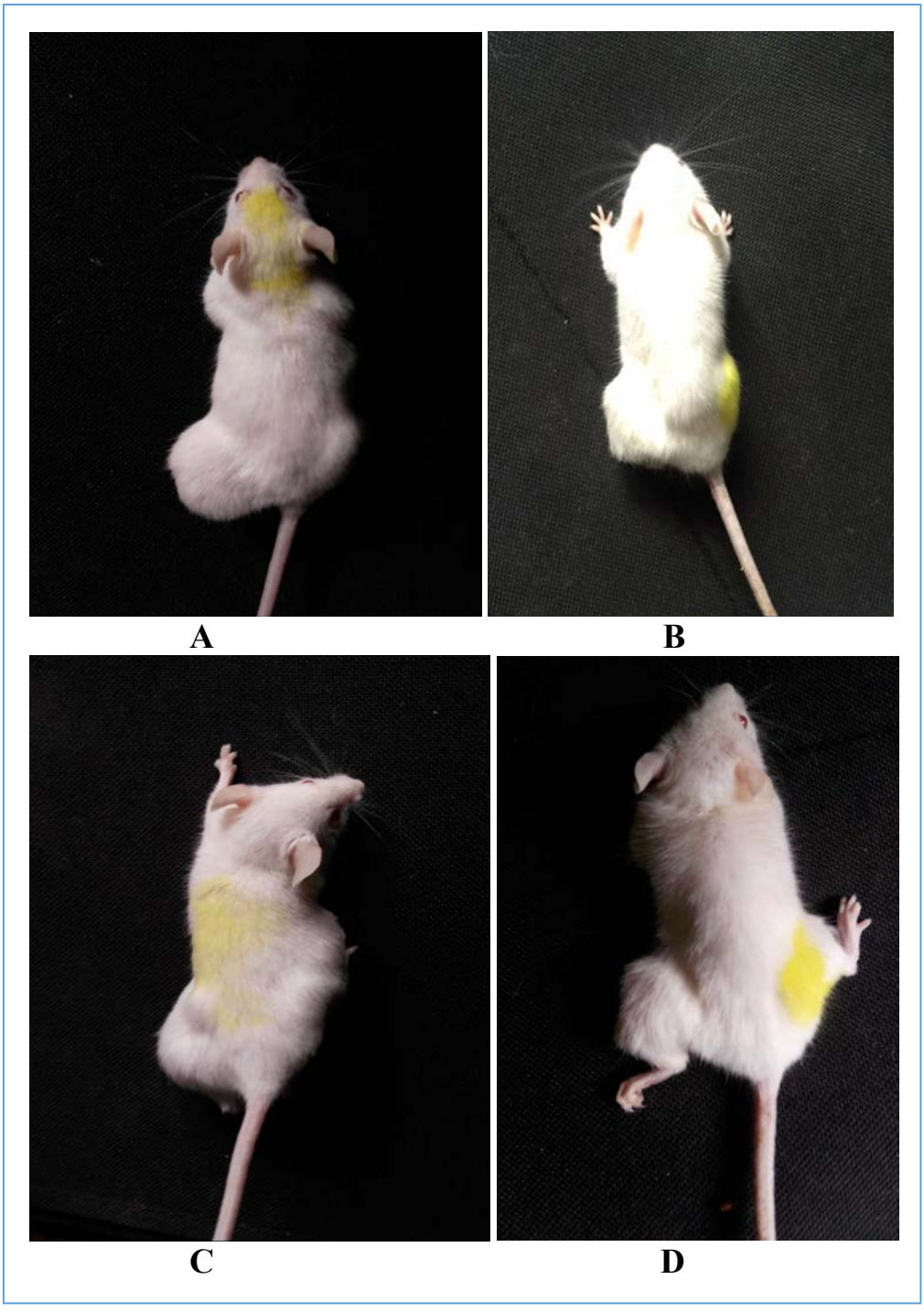


Table 8. Antitumor activity of ethanol extract of *S. stipitatum* on DLA induced solid tumor in mice

Treatment groups	**Tumor volume (cm3) ±SE**										
	0^{th} day	3^{rd} day	6^{th} day	9^{th} day	12^{th} day	15^{th} day	18^{th} day	21^{st} day	24^{th} day	27^{th} day	30^{th} day
Control	0.344 ±0.0977	0.478 ±0.105	0.625 ±0.170	0.978 ±0.175	1.696 ±0.093	1.921 ±0.122	2.493 ±0.192	2.819 ±0.125	3.079 ±0.198	3.123 ±0.095	3.174 ±0.123
Standard	0.164 ±0.066	0.250 ±0.119	0.254 ±0.092	0.291 ±0.051	0.337 ±0.121	0.523 ±0.110	0.612 ±0.110	0.634 ±0.132	0.695 ±0.063	0.726 ±0.074	0.738 ±0.076
Higher dose	0.225 ±0.044	0.321 ±0.131	0.431 ±0.091	0.551 ±0.068	0.600 ±0.130	0.749 ±0.123	0.789 ±0.111	0.830 ±0.086	0.870 ±0.051	0.910 ±0.157	0.941 ±0.143
Lower dose	0.209 ±0.019	0.412 ±0.136	0.600 ±0.071	0.620 ±0.118	0.742 ±0.198	0.891 ±0.175	0.952 ±0.105	0.982 ±0.152	1.100 ±0.052	1.265 ±0.152	1.389 ±0.173

Plate 2. Antitumor effect of ethanol extract of *S. stipitatum* on DLA-induced solid tumor in mice. A - Control, B - Standard, C - Ethanol extract of *S. stipitatum* (200 mg/kg), D - Ethanol extract of *S. stipitatum* (50 mg/kg)



4.5.2.2 EAC-induced ascites tumor model

The injection of EAC cell lines intraperitoneally into Swiss albino mice resulted in the development of ascites tumor. After 24 hrs of tumor implantation, the extracts and standard drug were given. The ethanol extracts were given orally for 10 days while cyclophosphamide, a standard drug, was administered intraperitoneally. The tumor progression was tracked on a regular basis, and the mortality pattern of the animals owing to tumor mass was noted. To determine the effect of the extract, the percentage increase in life span was determined.

Table 9. Number of EAC-bearing mice that survived in each group

Groups	**Days**									
	10	13	15	18	20	25	30	35	40	45
Control	6	5	5	4	3	2	0	0	0	0
Standard	6	6	6	6	5	3	3	2	0	0
200 mg/kg	6	6	6	6	6	4	3	2	1	0
50 mg/kg	6	6	6	6	6	3	1	0	0	0

Figure 11. Effect of ethanol extract of *S. stipitatum* on survival pattern of ascites tumor- bearing mice

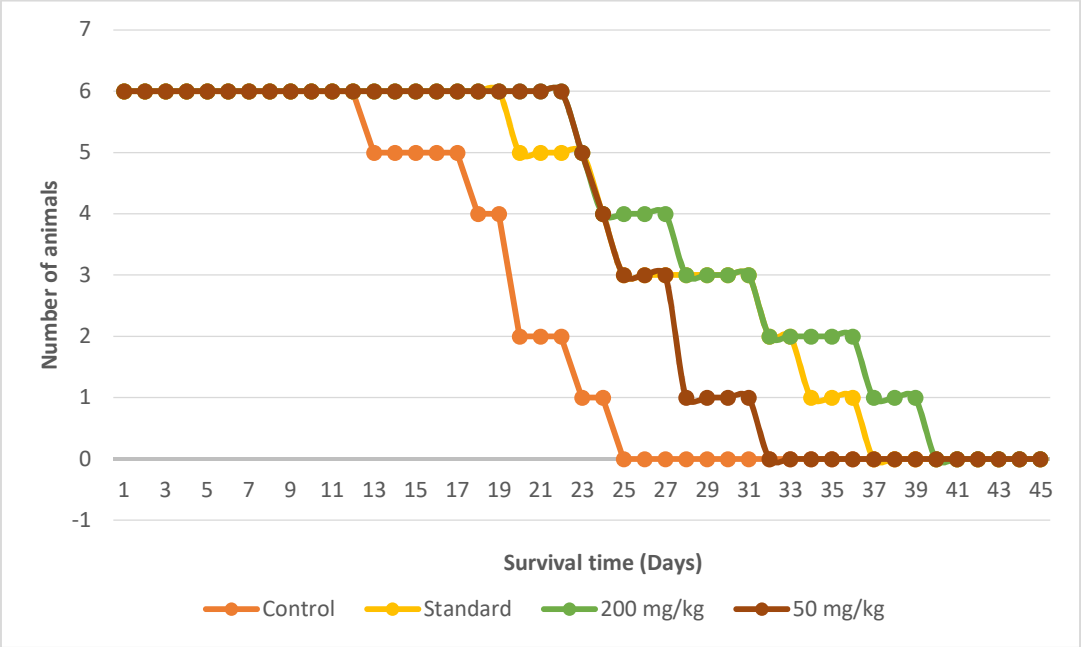
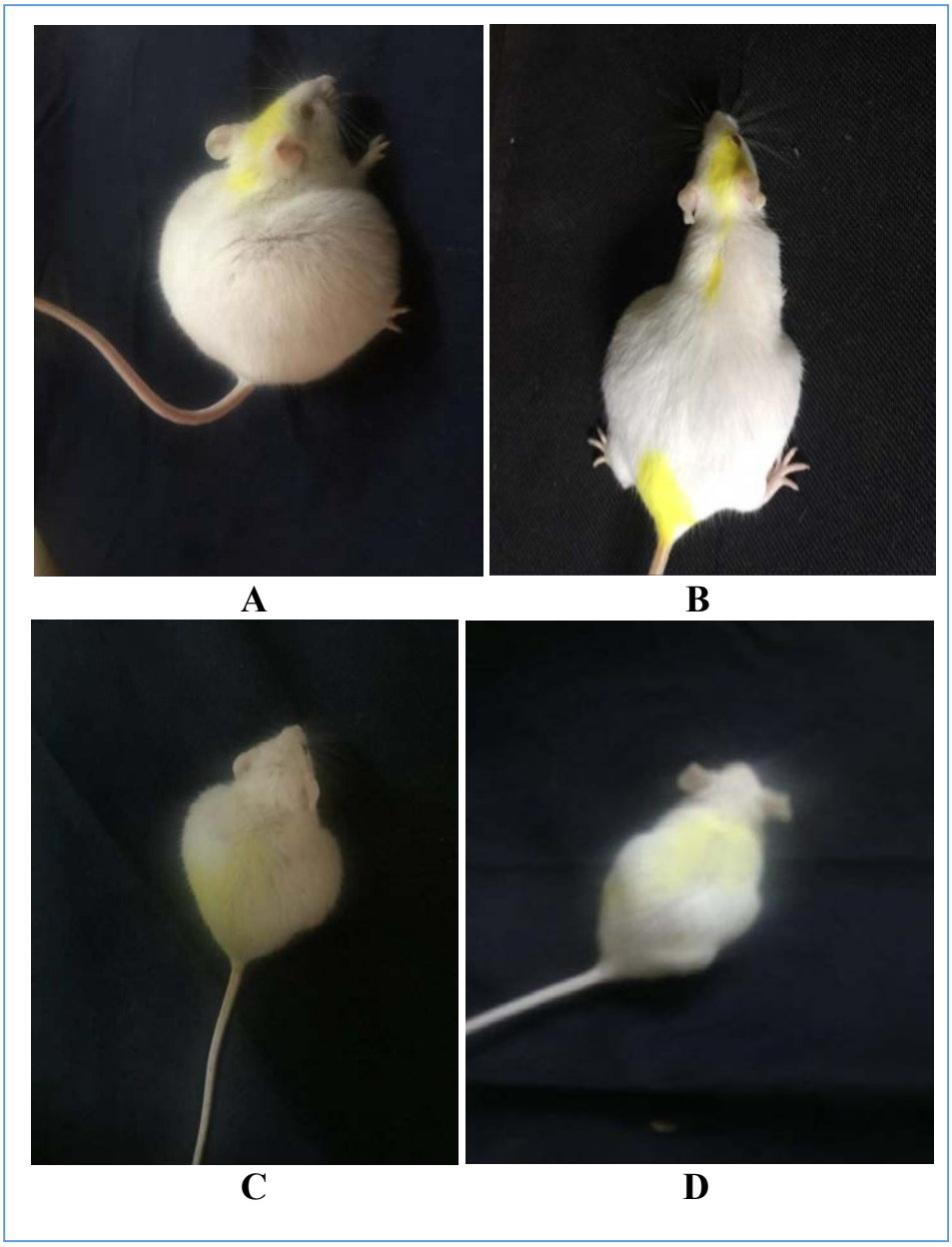


Table 10. Percentage increase in life span in comparison with control

Sl. No.	Group	Lifespan (± SE)	Percentage increase in life span (%)
1	Control	19.8 ±0.45	
2	Cyclophosphamide (10 mg/kg)	28.6 ±0.67****	44.4
3	200 mg/kg	30.7 ±0.58****	55.0
4	50 mg/kg	26.6 ±0.89****	34.3

Values are expressed as Mean ± SE, n = 6, **** p< 0.0001 compared to control considered as significant.

Plate 3. Antitumor effect of ethanol extract of *S. stipitatum* on EAC-induced ascites tumor in mice. A - Control, B - Standard, C - Ethanol extract of *S. stipitatum* (200 mg/kg), D - Ethanol extract of *S. stipitatum* (50 mg/kg)



4.5.3 In vivo antioxidant study

Effect of ethanolic extract of *S. stipitatum* treatment on antioxidant profile of blood in sodium fluoride induced animals

The blood of untreated control mice treated with sodium fluoride had substantially lower levels of catalase and superoxide dismutase activity than the normal reference animal group. In contrast to the NaF stress-induced control, antioxidant markers in blood were significantly elevated in the ethanol extract of *S. stipitatum* treated mice in a dose-dependent way. The pre-treatment effects were similar to those shown in mice treated with ascorbic acid (standard).

Table 11. Effect of ethanol extract of *S. stipitatum* on antioxidant profile of blood in NaF induced animals

Animal Groups	**Catalase (U/mg Hb)**	**SOD (mU/mg Hb)**
NaF alone	2.22 ±0.25	89.41 ±27.77
Normal	4.61 ±0.16****	506.33 ±14.17****
Standard	4.34 ±0.19****	260.75 ±20.10****
Low dose	3.32 ±0.07***	255.62 ±42.45****
High dose	3.96 ±0.06****	407.97 ±20.54****

Data represent the mean ± SE **** p< 0.0001, ***p< 0.001 compared to NaF considered as significant.

Table 12. Effect of ethanol extract of *S. stipitatum* on antioxidant profile of liver in NaF induced animals

Animal Groups	Catalase (U/mg protein)	SOD (U/mg protein)	GSH (nmols/mg protein)	GPx (U/mg protein)	GR (U/mg protein)	LPO (nmols/ mg protein)
NaF alone	1.98 ±0.12	2.29 ±0.06	5.86±0.26	34.63 ±0.98	81.61 ±0.65	3.63 ±0.25
Normal	2.18 ±0.14**	3.25 ±0.05****	11.15±0.76****	36.95 ±1.16**	83.6 ±1.1*	3.27 ± 0.15***
Standard	2.28 ±0.09***	3.51 ±0.17****	7.71 ±0.92**	40.89 ±1.16****	84.38 ±0.71**	2.81 ±0.18****
Low dose	2.17 ±0.03*	2.81 ±0.14****	9.02 ±0.95****	40.12 ±1.61****	85.46 ±1.81****	3.34 ±0.06****
High dose	2.30 ±0.10****	3.02 ±0.15****	7.49 ±0.76**	42.74 ±0.61****	79.22 ±1.70*	3.20 ±0.05***

Data represent the mean ± SE ****p< 0.0001, ***p<0.001, **p<0.01* p< 0.05 compared to NaF alone considered as significant

Effect of ethanolic extract of *S. stipitatum* treatment on antioxidant profile of liver in sodium fluoride induced animals

In the sodium fluoride induced untreated control group, sodium fluoride induction had a substantial influence on the antioxidant profile of the liver as evident from the decrease in reduced catalase, SOD activity, glutathione level,

glutathione peroxidase activity and GR activity. Despite sodium fluoride exposure, *S. stipitatum* ethanol extract pre-treated groups seemed to regain near-normal levels of these parameters in comparison with the control group. The standard treated with ascorbic acid had similar findings.

4.5.4 In vivo anti-inflammatory study

4.5.4.1 Acute carrageenan-induced model

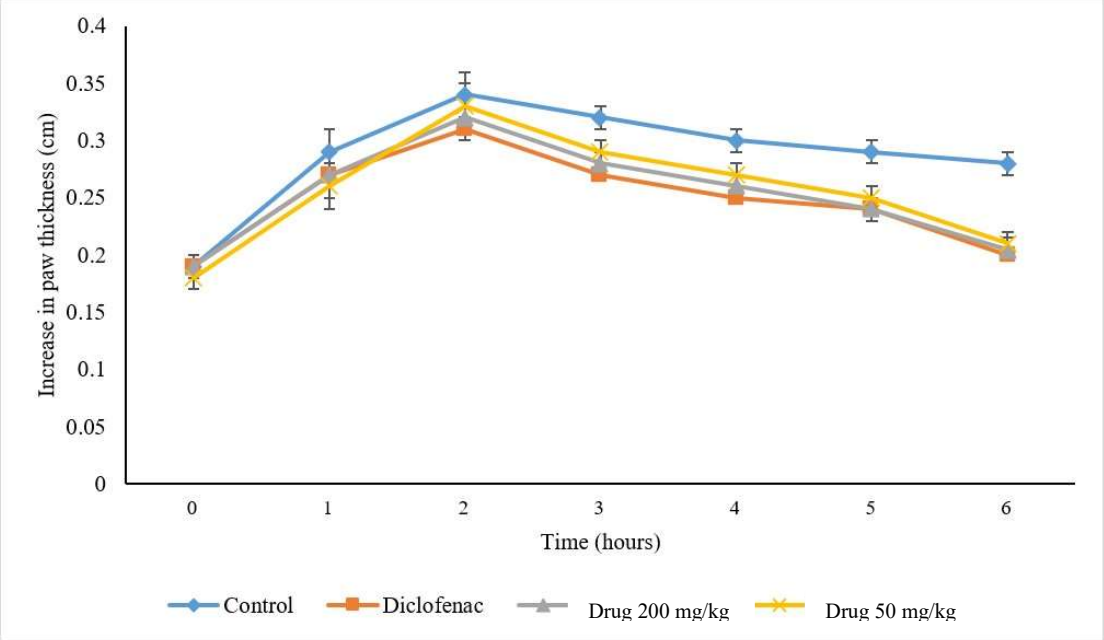
The ethanol extract of *S. stipitatum* significantly inhibits the acute inflammation induced by carrageenan. The extracts at concentrations 200 and 50 mg/kg reduced the paw thickness 83.33 and 66 % respectively as compared to control. The activity of standard reference drug Diclofenac at 10 mg/kg b. wt. showed 88.88% (Figure 12 and Table 13).

Table 13. Effect of ethanol extract of *S. stipitatum* on carrageenan- induced paw edema

Group	Initial paw thickness (cm)	Final paw thickness (cm)	Percentage inhibition (%)
Control	0.19 ±.01	0.28 ±.01	-
Diclofenac (10 mg/kg)	0.19 ±.015	0.20 ±.005**	88.88
SS Extract (200 mg/kg)	0.19 ±.01	0.205 ±.01**	83.33
SS Extract (50 mg/kg)	0.18 ±.012	0.21 ±.012**	66

Values are expressed as Mean ± Standard deviation (SD), n = 6, **p<0.01 compared to control considered as significant.

Figure 12. Paw thickness at each hour in carrageenan- induced model



4.5.4.2 Chronic formalin-induced model

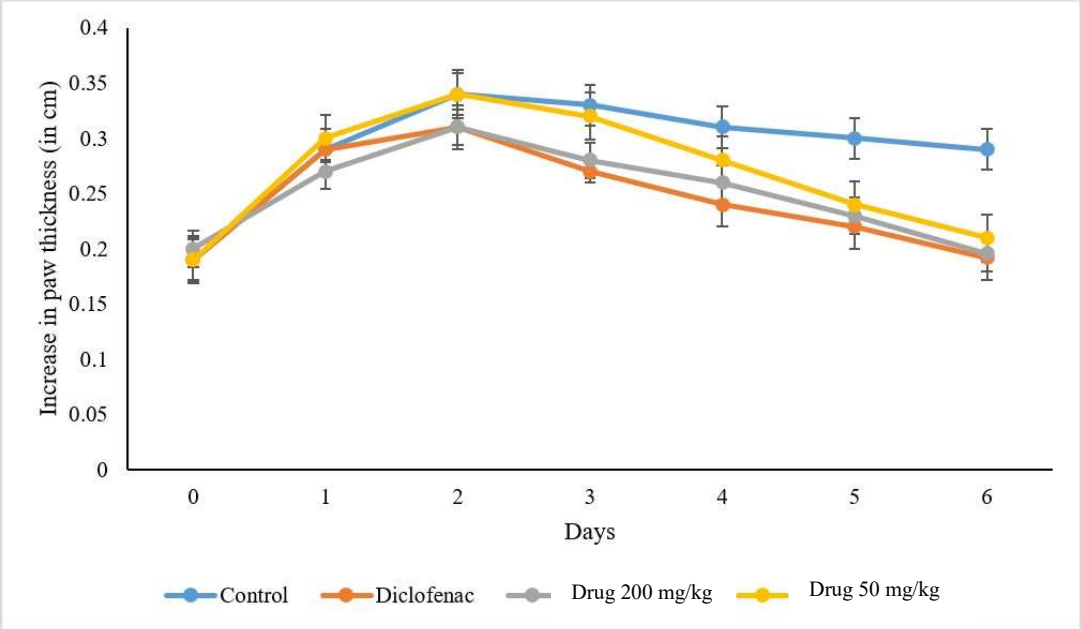
The ethanol extract of *S. stipitatum* significantly inhibits the chronic inflammation induced by formalin. The extracts at concentrations 200 and 50 mg/kg reduced the paw thickness 94 and 80% respectively as compared to control. The activity of standard reference drug Diclofenac at 10 mg/kg b. wt. showed 98% (Figure 13 and Table 14).

Table 14. Effect of ethanol extract of *S. stipitatum* in formalin-induced paw edema

Group	Initial paw thickness (cm)	Final paw thickness (cm)	Percentage inhibition (%)
Control	0.19 ±0.01	0.29 ±0.01	-
Diclofenac (10 mg/kg)	0.19 ±0.02	0.192 ±0.02****	98
SS Extract (200 mg/kg)	0.20 ±0.01	0.196 ±0.01**	94
SS Extract (50 mg/kg)	0.19 ±.01	0.210 ±.01**	80

Values are expressed as Mean ± SD, n = 6, **** p< 0.0001, ** p< 0.01 compared to control considered as significant.

Figure 13. Paw thickness at each day in formalin-induced model



4.6 Mycosynthesis of silver nanoparticles

A 1 mM aqueous solution of silver nitrate was made for the synthesis of silver nanoparticles. 30 mL of *S. stipitatum* aqueous extract and 270 mL of silver nitrate 1 mM aqueous solution were used in the reaction media. The reaction was then allowed to continue at room temperature for another 24 hrs. The reduction of silver nitrate to silver nanoparticles was clearly detected as the reaction mixture turned brown (Figure 14).

The mycosynthesized silver nanoparticles was characterized by UV- Vis spectroscopy, XRD, SEM and TEM.

Figure 14. The color change in synthesized AgNPs from *S. stipitatum*

4.6.1 UV-Visible Spectra analysis of silver nanoparticles

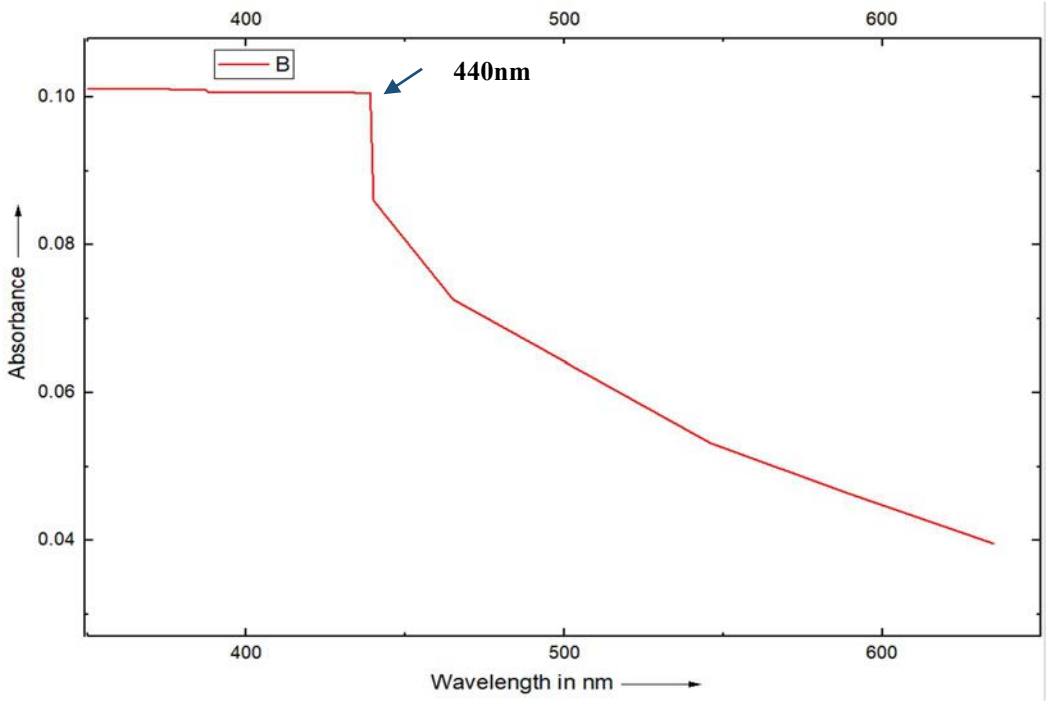
The UV–Visible spectra of the suspension showed typical surface plasmon resonance (SPR) around 440 nm which is a characteristic feature of silver nanoparticles.

Table 15. Result data of UV-vis spectra analysis of silver nanoparticles synthesized from ethanol extract of *S. stipitatum*

AU (440.00 nm)	AU (465.00 nm)	AU (546.10 nm)	AU (590.00 nm)	AU (635.00 nm)
0.0806	0.0726	0.0532	0.0463	0.0396

Absorbance (AU) at specific nm is given

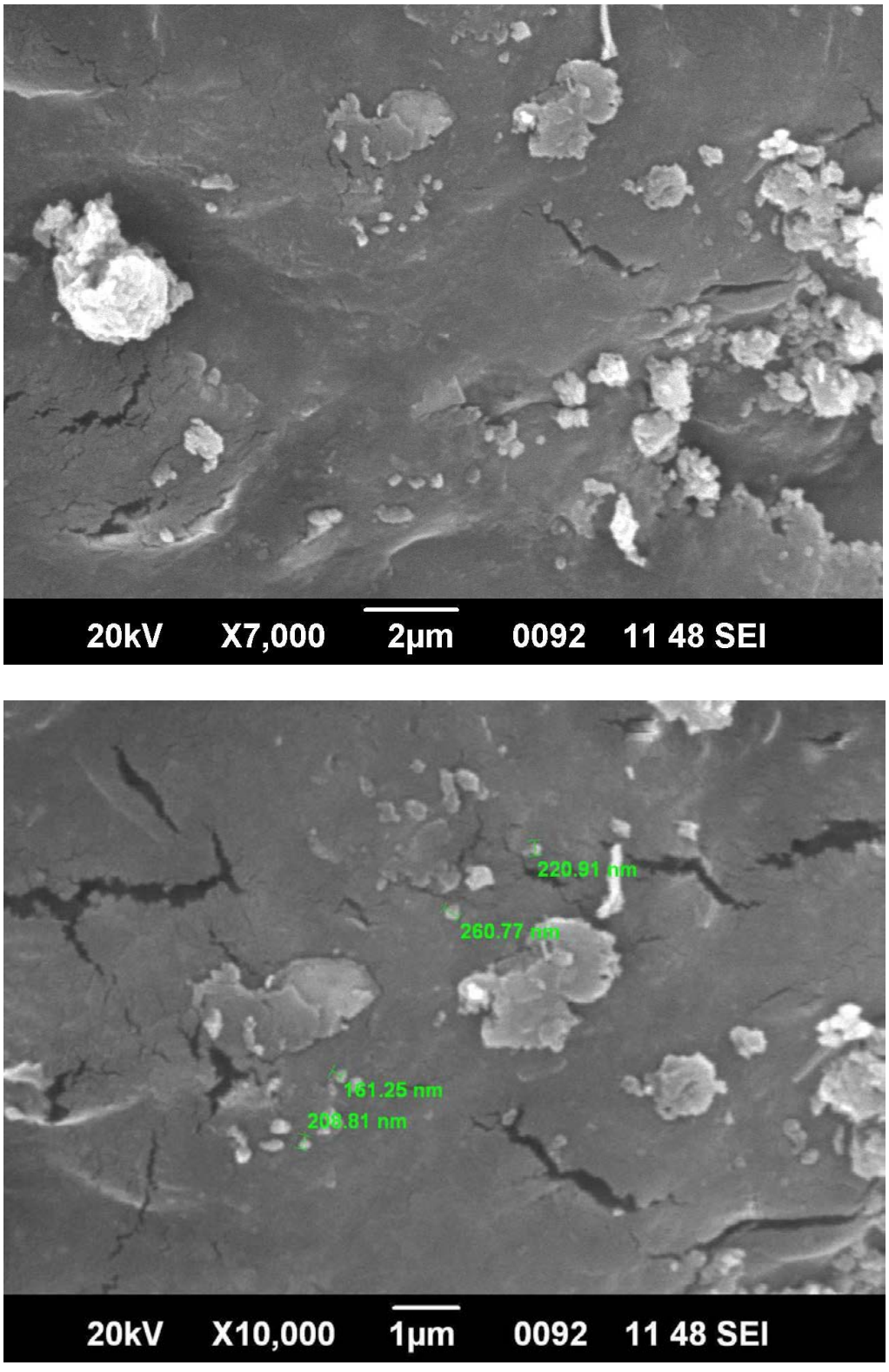
Figure 15. UV-Visible spectra of mycosynthesized silver nanoparticles from *S. stipitatum*



4.6.2 SEM analysis of silver nanoparticles

SEM analysis shows that the biosynthesized silver nanoparticles are spherical in shape, indicating that they were surface deposited silver nanoparticles. Monodispersity, shape, and particle size were all strongly reliant on AgNPs' function.

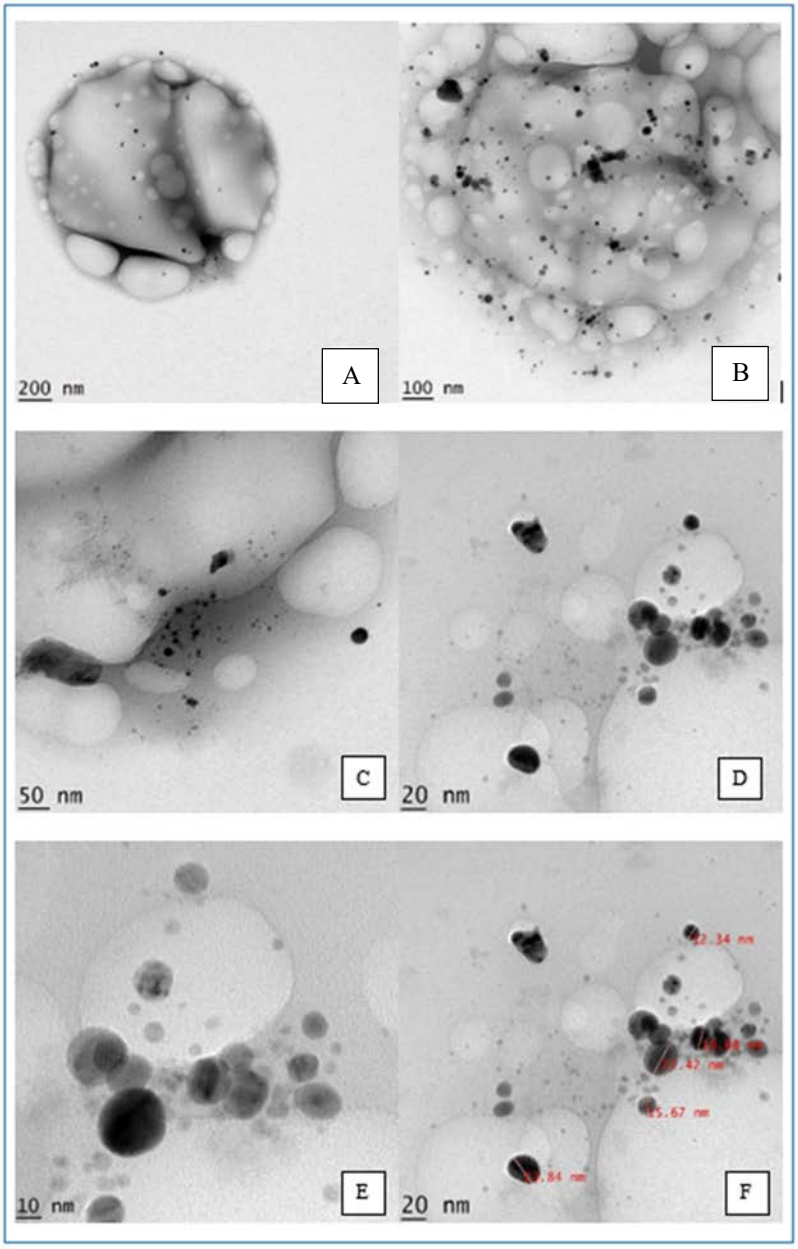
Figure 16. SEM micrographs of mycosynthesized silver nanoparticles from *S. stipitatum* (A- magnification 7,000 X, B- magnification 10,000 X)



4.6.3 TEM analysis of silver nanoparticles

TEM analysis provided further knowledge of the morphology and size of AgNPs. It shows the average size of the nanoparticles ranges from 12-28 nm (12.34, 15.67, 19.68, 23.84, 27.42).

Figure 17. TEM micrographs of mycosynthesized silver nanoparticles from *S. stipitatum* in various nm (A- 200 nm, B- 100 nm, C- 50 nm, D- 20 nm, E- 10 nm, F- 20 nm)



4.6.4 XRD analysis of silver nanoparticles

The crystalline structure of silver nanoparticles was revealed by X-ray diffraction patterns. Peaks at 2θ values of 38.148, 43.885, 64.488, and 77.398 correspond to crystal planes (1 1 1), (2 0 0), (2 2 0), and (3 1 1), respectively. The XRD spectrum verified the existence of silver nanoparticles.

Figure 18. X-ray diffraction pattern of mycosynthesized silver nanoparticles from *S. stipitatum*