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Chloroform leaf extract of *Salix alba* is a rich source of antimicrobial agents of enormous clinical importance

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Abstract:

Salix alba has been used from a long time as medicine. In this study a novel attempt was made to determine phytochemical composition and in vitro effects of chloroform leaf extract of Salix alba against Candida albicans (SN-2320), Candida tropicalis (SN-1982), Salmonella typhi (SN-0464) and Escherichia coli (SN-1224). Phytochemical analysis revealed the presence of phenols, Proteins, glycosides, steroids and alkaloids in different concentrations. In GC-Mass analysis, the major chemical compounds were Lignocerol (22.43%), Tetracosonal (17.17%), Cholesterol (9.63%) and Salicyl alcohol (7.86%). Biological activities carried out in terms of Minimum Inhibitory concentration, disc diffusion assay and growth curve studies revealed that the test extract has significant antimicrobial activity against Candida albicans, Candida tropicalis, Salmonella typhi and Escherichia coli. From the data, we concluded that chloroform leaf extract of Salix alba can be used as a raw material for future anti-fungal and anti-bacterial drug development.

Key words: *Salix alba*, Chloroform leaf extract, Phytochemicals, GC-Mass Analysis, Biological activity.

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Introduction:

Since ancient times, humans have used natural products for medicine. Plants are biochemical store houses that produce within their cells a range of complex substances with several active compounds that are used to cure many kinds of illnesses. White willow (*Salix alba*) belongs to genus *Salix* and family Salicaceae. *S. alba* ranges from prostrate shrub to large tree over 35m height. White willow which is also known as salicin willow has been used for medicine since long time [1]. Records suggest that *Salix alba* has been used since 6000 years ago in Mesopotamia and it was used to cure Assyrian, pain and inflammation, Sumerian, arthritis, malaria, various haemorrhages, gout, neuralgia and intestinal diseases as an antipyretic, antibacterial, haemostatic, sedative and antihelminthic agent. A brew of willow leaves has been prescribed to ease the unbearable pains in childbirth. For pain and inflammation white willow has been continued for use [2]. *Salix alba* has been used for the treatment of different kind of pain, including back pain, toothache, menstrual cramps and rheumatic pain. It has also been used for fever, headache and sore throat connected with upper respiratory tract infections and influenza [3]. Even though this plant has been used since long for medicinal purpose, study for its medicinal properties is still needed. Therefore the objective of the present study is to investigate antimicrobial activity of chloroform leaf extract of *S. alba.*

Candida is a genus of yeast. Some species are harmless endosymbionts of hosts including humans but other species or harmless species in the wrong location can cause diseases. *Candida albicans* can cause (candidiasis or thrush) in humans and other animals, especially in immunocompromised patients. Essentially all areas of the human gastrointestinal tract can harbour *Candida.* While as *Candida tropicalis* is a casual agent of opportunistic oral and genital infections in humans. [4, 7]

Bacteria are unicellular prokaryotic organisms characterised by the lack of a membrane bound nucleus and membrane-bound organelles. Bacteria are remarkably adaptable to diverse environmental conditions. They are found in the bodies of all living organisms and on all parts of earth [8, 9]. Salmonellosis is an infection with the bacteria called *Salmonella. Salmonella* germs have been known to cause illness for over 100 years. They were discovered by American scientist named salmon, for whome they are named. Most persons infected with salmonella develop diarrohea, fever and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days [10]. *Escherichia coli* bacteria normally lives in intestinal of the people & animals. Most *E. Coli* are harmless and actually are an important part of a healthy human intestinal tract. However, some *E. coli* are pathogenic meaning they can cause illness, either diarrohea or illness outside of the intestinal tract. The types of *E. coli* that can cause diarrohea can be transmitted through contaminated water or food, or through contact with animals or persons [11, 12]

Material & Methods

Collection and preparation of plant material

Salix alba leaves were collected from Plant nursery of Duroo sopore Kashmir. Department of forestry, District Baramulla Jammu & Kashmir is taking care of this nursery.

Collection of samples was done in September-October 2013, because in this season leaves are completely developed. A voucher specimen of 'Lib232-SA5' was stored in laboratory for further exercise. Leaves were washed, dried, grinded and extracted with chloroform in Soxhlet apparatus for 72h, then filtered with Whattman filter paper and filtrate was evaporated to dryness. After clearance of biosafety and ethical committee of the institute, the extract was used for phytochemical and antimicrobial investigation.

Phytochemical analysis

Chloroform extract was subjected to various phytochemical tests to find out major constituents. Tests were performed for glycodies (keller-Killiani Test), tannins (Braemer's test), steroids, terpenoids (Salkowski test), alkaloids (Mayer's/ Wagner's test) and Anthraquinones [13, 16].

GC-MS analysis

By using a Shimadzu 2010 gas chromatograph fitted with an AB-Wax column GC-MS analysis of the extract was carried out. Helium was used as carrier gas. Sample (0.2ml) was injected in the splitless mode. The chemical component from the extract was identified by comparing the retention time of the chromatographic peaks with those of authentic compound using the WILEY8.LIB and NISTO5s.

Strains and growth media

Clinical Isolates of *Candida albicans (SN-2320)*, *Candida tropicalis (SN-1982)* used in this study were collected from Department of Microbiology, Vardhaman Mahavir Medical College, New Delhi, and bacterial isolates *Salmonella typhi (SN-0464)* Gram negative and *Escherichia coli (SN-1224)* Gram positive were collected from Holy Family Hospital, New Delhi India. The working fungal strains where maintained on YEPD slants containing 2% glucose, 2% peptone and 1% yeast extract maintained at -20°c [17] and bacterial strains where maintained on 2% nutrient agar slants and were subcultured twice prior to testing to ensure viability and purity [18]. All media constituents were obtained from Hi-Media (India). All chemicals and solvents were of analytical grade and were obtained from Glenmark (India). For all experimental studies the yeast cells were maintained on the yeast extract-peptone-dextrose (YEPD) medium and bacterial cells on nutrient agar medium at 37°c.

Determination of Minimum Inhibitory Concentration

MICs of the strains were determined using a broth microdilution method. Cells were resuspended in a 0.9% normal saline solution to give an optical density at 600nm (A_{595}=0.1). The diluted cell suspensions were added to the wells of round-bottomed 96 well microtiter plates containing equal volumes of medium and different concentrations of test extract [18, 19]. A drug free control was also included. At the end MIC test was evaluated visually and defined as the lowest extract concentration that showed significant inhibition of growth compared to the controls.

Filter Disc Assay

Filter disc assay was performed according to the standard guidelines (M2-A7) of the national committee for clinical laboratory standards (NCCLS), using a modified Kirby-Bauer disc diffusion method [20]. All the organisms were stored at -20°C until use. Cells were grown at 37°C in YEPD broth and nutrient agar (approximately 10^5 cells/ml) and were passaged at least twice on solid agar. Broth cultures were swabbed onto agar to achieve a lawn of confluent growth. Stock solutions of the test extract were prepared in 1% chloroform. Paper discs impregnated with different extract concentrations were placed on each plate. One disc impregnated with 1% chloroform was placed in the centre of the plate that served as solvent control. The plates were incubated at 37°C for 48 hrs. The diameter of the zone of inhibition was recorded in millimetres, after 48 hours [21].

Growth Curve Studies

A growth curve is an empirical model of the evolution of a quantity over time [22]. This experiment carried 10^6 cells {optical density A_{595}=0.1} of strains which were grown aerobically in automated shaker set at 37°C until stationary phase. Growth was followed turbidometrically at 595nm using

spectrophotometer. Required concentrations of test extract were added to culture. The growth rates of cells alone and with inhibitor were performed. Optical density was recorded for each concentration against time (hrs). The growth rate is equivalent to slope log (optical density) versus time duration the exponential phase [23].

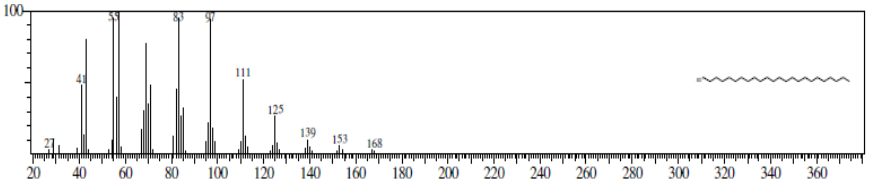
Results & Discussion

Phytochemicals & GC-Mass analysis

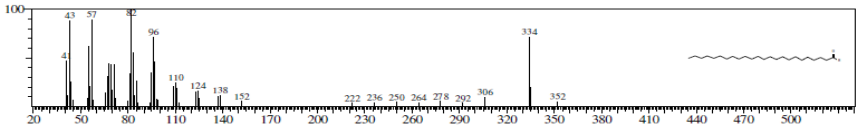
Phytochemical analysis revealed the presence of Phenols, proteins, glycosides, steroids, and alkaloids in different concentration while as starch, tannis and anthraquinones were fund absent (Table 1). In GC-Mass analysis 48 chemical compounds were found out of which 4 were found in the major concentration marked with red colour in (Table 2), viz., Lignocerol (22.43%), Tetracosonal (17.17%), Cholesterol (9.63%) and Salicyl alcohol (7.86%). The interpretation and nomenclature of phytochemicals is based on the molecular formula, molecular weight, retention time and percentage of presence.

Fragmentation pattern of four major Chemical Compounds.

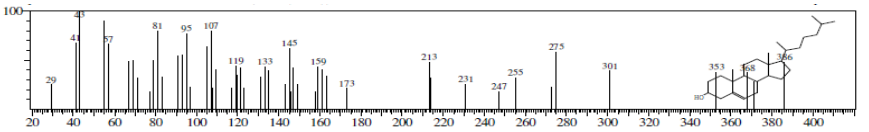
1. Lignocerol ($C_{24}H_{50}O$), M. Weight = 354, R. Time= 25.97, % of presence=22.43



2. Tetracosonal ($C_{24}H_{48}O$), M. Weight=352, R. Time=25.24, % of presence=17.17



3. Cholesterol ($C_{27}H_{46}O$), M. Weight=386, R. Time=33.73, % of presence=9.63



4. Salicyl Alcohol ($C_7H_8O_2$), M. Weight=124, R. Time=23.93, % of presence=7.86

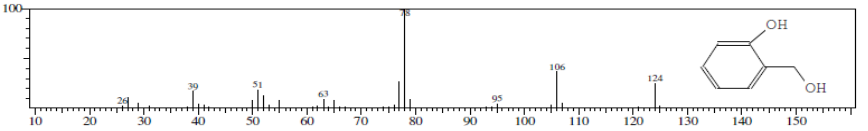


Table 1: Phytochemical Screening of various compounds

Constituents	Observation
Phenols	Present
Proteins	Present
Glycosides	Present
Steroids	Present
Alkaloids	Present
Tannins	Absent
Starch	Absent
Anthraquinones	Absent

Table 2: List of detected compounds in the successive chloroform leaf extract of *Salix alba.*

S. No	Name of Compound	Molecular Formula	Molecular Weight	Retention Time	% of Presence
1	Lignocerol	$C_{24}H_{50}O$	354	25.97	22.43
2	Tetracosanal	$C_{24}H_{48}O$	352	25.24	17.17
3	Cholesterol	$C_{27}H_{46}O$	386	33.73	9.63
4	Salicyl Alcohol	$C_7H_8O_2$	124	23.93	7.86
5	Vitamin E	$C_{29}H_{50}O_2$	430	29.61	4.02
6	Stearyl aldehyde	$C_{18}H_{36}O$	268	22.6	3.87
7	1-Heptacosanol	$C_{27}H_{56}O$	396	28.82	3.77
8	1,2-Epoxyoctadecane	$C_{18}H_{36}O$	268	41.01	3.39

9	Octadecanal	$C_{18}H_{36}O$	268	27.83	2.15
10	Isomenthol	$C_{10}H_{20}O$	156	16.74	1.79
11	2-Nonadecanone	$C_{19}H_{38}O$	282	29.11	1.58
12	Pentatriacontane	$C_{35}H_{72}$	492	20.49	1.47
13	Hexadecanoic acid	$C_{16}H_{32}O_2$	256	15.33	1.37
14	E-15-Heptadecenal	$C_{17}H_{32}O$	252	13.51	1.16
15	Ethyl docosanoate	$C_{24}H_{48}O_2$	368	15.56	1.15
16	Hexadecyl trifluoroacetate	$C_{18}H_{33}F_3O_2$	338	17.42	1.04
17	1-Heptacosanol	$C_{27}H_{56}O$	396	21.88	0.97
18	Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292	17.1	0.95
19	Pentatriacontane	$C_{35}H7_2$	492	24.76	0.88
20	Dotriacontyl heptafluorobutyrate	$C_{36}H_{65}F_7O_2$	662	23.63	0.81
21	Lignoceric alcohol	$C_{24}H_{50}O$	354	19.27	0.73
22	Myricyl alcohol	$C_{30}H_{62}O$	438	32.97	0.73
23	2,7-Dibutoxy-fluoren-9-one oxime	$C_{21}H_{25}NO_3$	339	19.04	0.69
24	Cerylic alcohol	$C_{26}H_{54}O$	382	34.76	0.63
25	Pentadec-1-ene	$C_{15}H_{30}$	210	11.22	0.62
26	9-Eicosyne	$C_{20}H_{38}$	278	14.03	0.62
27	cis-9-Hexadecenal	$C_{16}H_{30}O$	238	26.43	0.55
28	Spathulenol	$C_{15}H_{24}O$	220	21.32	0.54
29	Isolongifolene, 4,5-dehydro-	$C_{15}H_{22}$	202	18.76	0.53
30	Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	292	23.8	0.52
31	Tridecene-1	$C_{13}H_{26}$	182	8.62	0.51
32	Ambreinolide(cis-A/B)	$C_{17}H_{28}O_2$	264	25.57	0.47
33	Octadecyl aldehyde	$C_{18}H_{36}O$	268	24.12	0.46
34	3,5-Di-tert-butylphenol	$C_{14}H_{22}O$	206	10.31	0.44
35	Ethyl 9,12,15-octadecatrienoate	$C_{20}H_{34}O_2$	306	17.25	0.41
36	Methyl heptadecyl ketone	$C_{19}H_{38}O$	282	26.17	0.39
37	Heneicosane	$C_{21}H_{44}$	296	18.34	0.32
38	Nonylcyclopropane	$C_{12}H_{24}$	168	5.64	0.29
39	Methyl heneicosanoate	$C_{22}H_{44}O_2$	340	14.9	0.28
40	Linolenic acid	$C_{18}H_{30}O_2$	278	16.63	0.28
41	11-Tetradecynyl acetate	$C_{16}H_{28}O_2$	252	17.66	0.28
42	2,5-Dimethoxybenzyl acetate	$C_{11}H_{14}O_4$	210	15.75	0.27
43	Palmitaldehyde	$C_{16}H_{32}O$	240	19.71	0.27
44	Citraconic anhydride	$C_5H_4O_3$	112	3.36	0.25
45	2,6-Cyclooctadien-1-ol	$C_8H_{12}O$	124	3.95	0.25
46	Guaiacylacetone	$C_{10}H_{12}O_3$	180	13.12	0.22
47	Limonene oxide, cis-	$C_{10}H_{16}O$	152	10.73	0.21
48	1-Nonadecene	$C_{19}H_{38}$	266	13.29	0.21

Biological Activity

Minimum inhibitory concentration & filter disc assay

For some fungal and bacterial species levelling from 800 to 1600µg/ml Leaf extract of *S. alba* displayed considerable MICs. Antifungal activity of the extract was studied against *Candida albicans (SN-2320)*, *Candida tropicalis (SN-1982)* at 3 different concentrations 400 mg/ml, 800 mg/ml and 1200mg/ml and antibacterial activity was studied against *Salmonella typhi (SN-0464), Escherichia coli (SN-1224)* at 4 different concentrations 200 mg/ml, 400 mg/ml, 600 mg/ml & 800 mg/ml respectively, calculations are listed in (Table 3) and revealed in (Figure 1a, 1b, 1c & 1d). Comparison between antifungal and antibacterial activities of the different extract concentrations and standard drug is shown in (Fig. 2 & Fig. 3). Observations have shown that the chloroform leaf extract of *S. alba* has significant antifungal and anbacterial activity. At 4mg/ml extract exhibited minimum inhibitory concentration against fungal species & at 2mg/ml for bacterial species. However the percentage varied with the concentration of the test extract.

Table 3: Antifungal & antibacterial screening data for different test extract concentration and also ketoconazole & Neomycin.

	Zone of Inhibition (mm)	
Concentration of extract	***Candida albicans (SN-2320)***	***Candida tropicalis (SN-1982)***
400 mg/ml	4	5
800 mg/ml	7	6
1200 mg/ml	10	12
ketoconazole (100µg/ml)	**14**	**15**
Control 1% Chloroform	--	--
Concentration of extract	***Salmonella typhi (SN-0464)* (Gram –ve)**	***Escherichia coli (SN-1224)* (Gram +ve)**
200mg/ml	2	3
400mg/ml	6	5
600mg/ml	8	8
800mg/ml	10	9
Neomycin (100µg/ml)	**16**	**15**
Control 1% Chloroform	-	-

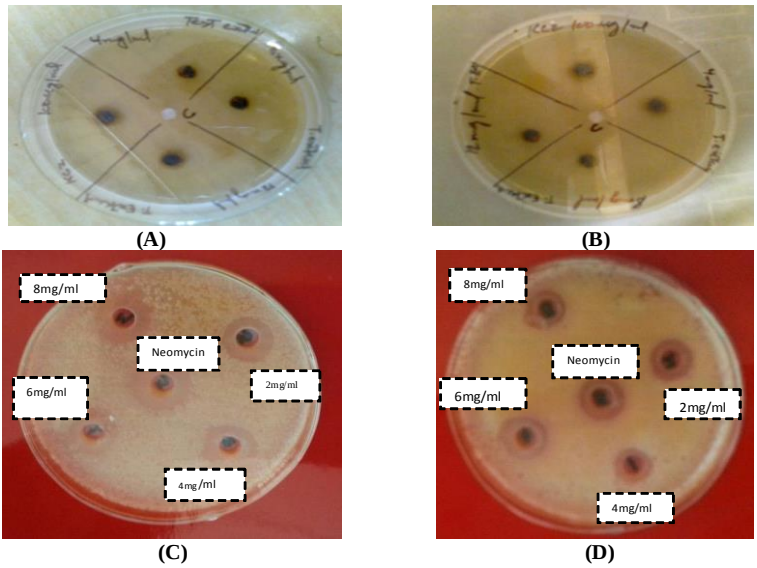


Figure 1: Filter disc assay of the test extract against **(A)** *Candida albicans (SN-2320)* **(B)** *Candida tropicalis (SN-1982)* ***(C)*** *Salmonella typhi (SN-0464)* ***(D)*** *Escherichia coli (SN-1224)*

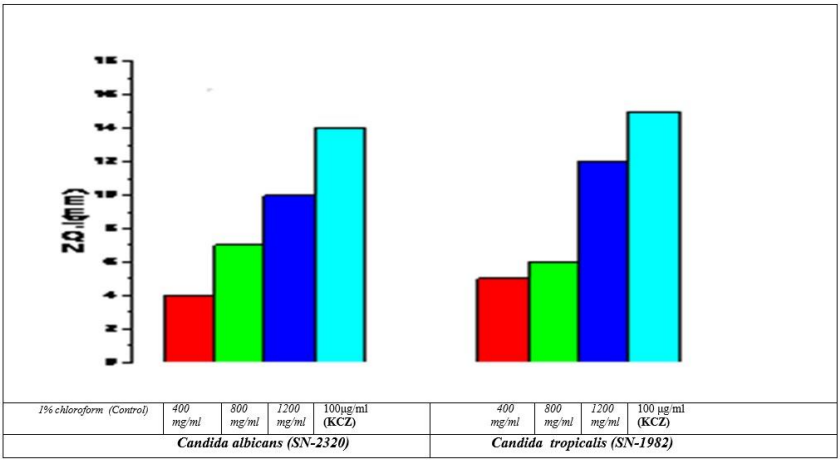


Figure 2: Bar diagram showing comparison between antifungal activities of Chloroform leaf extract *of S. alba* at different concentrations and standard antifungal drug against (a) *Candida albicans* (b) *Candida tropicalis*

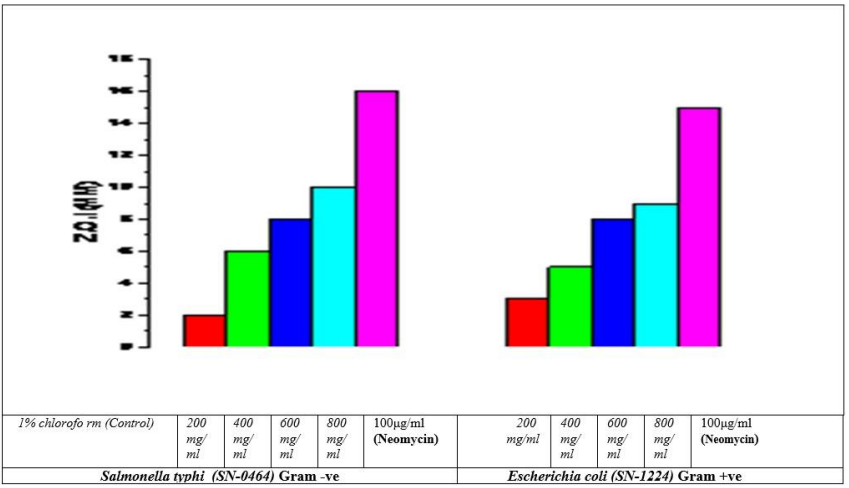
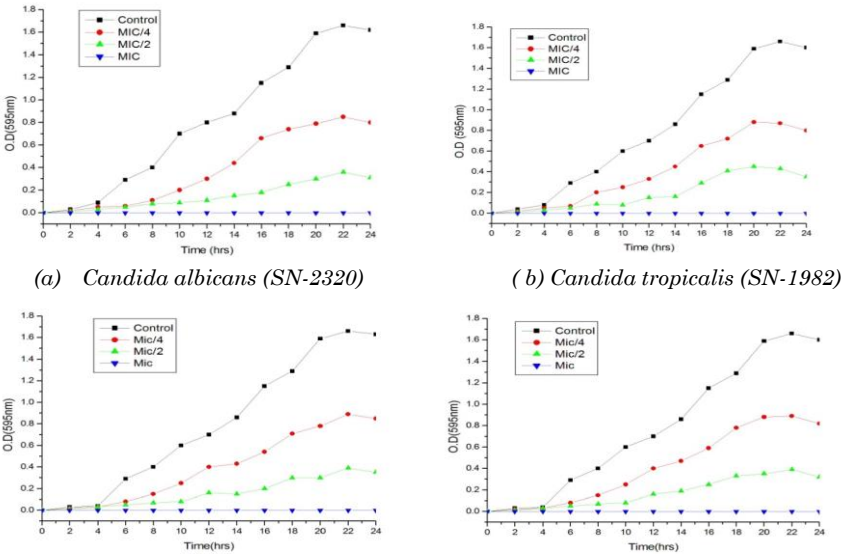


Figure 3: Bar diagram showing comparison between antibacterial activities of Chloroform leaf extract of *S. alba* at different concentrations and standard antibacterial drug against (c) *Salmonella typhi (SN-0464)* (d) *Escherichia coli (SN-1224)*

Growth Curve Studies

At different concentrations of chloroform leaf extract of *S. alba* growth curve of the fungal & bacterial Species was investigated. Figure 4a, 4b & 4c, 4d with dissimilar concentrations of chloroform leaf extract of *S. alba* showed different effect on growth pattern of *Candida albicans (SN-2320)*, *Candida tropicalis (SN-1982)* and *Salmonella typhi (SN-0464) Escherichia coli (SN-1224).* With the lag phase of 4 hrs control cells showed a normal growth & active exponential phase in 8-10 hrs before reaching last phase. The culture reached the stationary growth phase after 16 hrs in case of control cells as indicated by Optical density which showed normal curve, initially which showed lag stage, then exponential stage and at last a stationary stage. Increase in the concentration of the extract showed decrease in growth with concealed and deferred exponential phase in comparison to control. At minimum inhibitory concentration values almost

complete stopping of growth was observed which is indicated by flat line.



(a) Candida albicans (SN-2320) *(b) Candida tropicalis (SN-1982)*

(C) *Salmonella typhi (SN-0464)* *(d) Escherichia coli (SN-1224)*

Figure 4: Determination of different concentrations of chloroform leaf extract of *S. alba* on growth curve pattern against absorbance at 595nm (hrs) shown complete inhibition of growth at MIC values against (a) *Candida albicans (SN-2320)* (b) *Candida tropicalis (SN-1982)* (c) *Salmonella typhi (SN-0464) (d) Escherichia coli (SN-1224)*

Discussion

Natural resources have provided an unparalleled source of chemical scaffolds with diverse biological activities and have profoundly impacted antimicrobial drug discovery. The potential for developing antimicrobials from plants seems rewarding, as it will lead to the development of phytomedicine to act against different microbes. Our findings provide an idea for expanding the utility of plant active principals as antimicrobial agents. We have found that chloroform leaf extract of *S. alba* exhibits antifungal & antibacterial activity by Filter Disc Assay, and Growth Curve Study against two different fungal isolates and two bacterial isolates. Recently

obtained clinical isolates were found clearly sensitive to the test extract at varying extents. Test extract displayed considerable MIC against different fungal & bacterial isolates ranging from 800 to 1600 µg/ml. The extract was found highly active against *Candida albicans (SN-2320)*, *Candida tropicalis (SN-1982), Salmonella typhi (SN-0464) and Escherichia coli (SN-1224).* Growth kinetic studies as a function of varied concentrations also follow the same pattern. MIC/4 treated cells showed depressed growth curve with clearly differentiated phases. While as MIC/2 treated cells showed suppressed and delayed exponential phase. At MIC value the curve declined to flat line showing almost complete death of cell growth in both fungal & bacterial isolates. On solid media (filter disc assay) effective inhibition of growth of fungal isolates and bacterial isolates by test extract was found to increase in concentration dependent manner.

Conclusion

Chloroform leaf extract of *S. alba* has shown clear antimicrobial activity in both solid and liquid media. This work is an additional effort to the development of new therapeutic agent which is less toxic and prevents drug resistance. Further investigation and experimentation needs to be done, which is very essential & important and may help to facilitate its application as future antimicrobial agent.

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