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Phytochemical, GC-MS Analysis and Antibacterial Activity of Bioactive Compounds of Petroleum Ether Leaf Extracts of *Salix viminalis*

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Abstract: ***Medicinal Plants have been used for centuries as remedies for human illness. The objectives of the study was to isolate and analyze phytochemical constituents of Petroleum ether leaf extracts of Salix viminalis and their antibacterial activity against Escherichia coli SN 1224 (Gram -ve), Salmonella typhi SN 0464 (Gram –ve) and Staphylococcus aureus SN 1175 (Gram +ve). Preliminary phytochemical screening of the extract was carried out according to the standard methods, which showed presence of glycosides, phenols, alkaloids, terpenoids and flavonoids. GC-MS analysis showed forty seven chemical compounds out of which Nonadecyl trifluoroacetate (10.72), Tetracosanal (9.01), Cholesterol (8.11) and Cholest-4-en-3-one (8.02), Pentatriacontane (5.41) were found in major concentration. Petroleum ether leaf extracts of Salix viminalis has shown significant antibacterial activity against all the strains used in this study. Activity was measured in terms of minimum inhibitory concentration, disc diffusion assay and growth curve study. Antibacterial results revealed that the Petroleum ether leaf extracts of Salix viminalis can be used as a raw material for the future anti-bacterial drug.***

Keywords: *Salix viminalis* leaves, Petroleum ether, GC-MS, Antibacterial Activity, Phytochemical analysis.

1. Introduction

Medicinal Plants have been used for centuries as remedies for human illness. The plant kingdom still holds many species of plants which contain substances of medicinal values which are yet to be discovered [1]-[4]. Studies of the adverse effects of these herbal medicines and establishment of a good correlation between biomarkers and plants are essential for ensuring the efficiency and quality of the herbal medicines. *Salix viminalis* L. (White Willow) belongs to the family *Salicaceae*, this plant is native to Asia, North America, central and southern Europe. This plant has been used since ancient times for the health benefits [5]-[9]. This plant is commonly used in the treatment of arthritis, gout, malaria and intestinal diseases as an antipyretic, anti-inflammatory, antimicrobial, haemostatic, sedative and antihelminthic agent [10]-[11]. The fresh and clean bark of *S, viminalis* contains Salicin which gets decomposed into salicylic acid (which is closely related to aspirin) in human body [12]. Leaves juice is being used for astringent, expectorant, laxative: useful in fevers, tremors in the limbs, muscular pain, ophthalmia and enlargement of spleen [13]. Leaves of this plant contained flavonoids in major concentration, phenols, glycosides, alkaloids and terpenoids have also been already reported [14]. Ethanolic extract from the *Salix* has been reported to contain significant antioxidant and hepatoprotective property [15].

Bacteria are single celled, microscopic organisms which are found on most materials and surfaces and are often maligned as the causes of human and animal diseases (like *leptospira*, which causes severe disease in livestock). Bacteria are having immense importance because of their extreme flexibility, capacity for rapid growth and reproduction. Some of them make their own food from sunlight-Like plants, some are scavengers (share the environment around them) and some are warriors (they attack other living things) [16]-[17].

Escherichia coli bacteria were discovered by Theodor Escherich the German bacteriologist in 1885 in the human colon [18]. These are free living organisms more than 700 serotypes of *E. coli* have been identified. Most of the *E. coli* does not cause diseases, while as some cause infections other than gastrointestinal infections such as urinary tract infections [19]-[20]. Salmonellae are ubiquitous human and animal pathogens, and salmonellosis, a disease that affects an estimated 2 million Americans each year, is common throughout the world. *Salmonella typhi* is a food and water borne pathogen that can be easily disseminated in population [21]-[22]. Salmonellosis ranges clinically from the common salmonella gastroenteritis (diarrhea, abdominal cramps, and fever) to enteric fevers (including typhoid fever) which are life-threatening febrile systemic illness requiring prompt antibiotic therapy. Focal infections and an asymptomatic carrier state occur. The most common form of salmonellosis is a self-limited, uncomplicated gastroenteritis.

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterised by individual cocci, which divide in more than one plane to form grape-like clusters. To date, there are 32 species and eight sub-species in the genus Staphylococcus, many of which preferentially colonise the human body [23], however Staphylococcus aureus and Staphylococcus epidermidis are the two most characterised and studied strains Staphylococcus aureus is a major pathogen of increasing importance due to the rise in antibiotic resistance [24]

All these isolates are harmful in one way or the other so it is very important to check the antibacterial activity of Petroleum ether leaf extracts of *Salix viminalis,* very less study has been has been done on the *S. viminalis* leaves and the mode of action is not given yet or clearly understood. Thus the purpose of this study is to examine antibacterial action of Petroleum ether leaf extracts of *Salix viminalis* leaves against some *bacterial* species. Bioactivity of

Petroleum ether leaf extracts of *Salix viminalis* is to destroy *bacterial* cells which were screened by MIC, growth curve studies and filter disc assay.

2. Materials and Methods

2.1 Sample Collection and Authentication

S. viminalis leaves were collected from Duroo Sopore plant nursery. This nursery is affiliated to department of forestry district Baramulla Jammu and Kashmir, India and is well known for different species of *Salix*. Samples were collected in September-October 2012, in this season leaves are full grown, matured and modified. An authenticated voucher specimen of 'Lib232-SV2' was stored in laboratory for further investigation.

2.2 Preparation of Plant Material

150 g leaves of *S. viminalis* were washed, air-dried, coarsely powdered and was extracted with 550 ml of petroleum ether solvent by using Soxhlet apparatus. After extraction the sample was kept in dark for 72 h with intermittent shaking. Then the solvent was evaporated under reduced pressure using vacuum rotary evaporator and to obtain viscous semi solid masses.

2.3 Phytochemical Screening

Petroleum ether leaf extracts of S. viminalis were tested for alkaloids, phenolic compounds, flavonoids, saponins, steroids, sugars, tannins, Anthraquinones and amino acids. Phytochemical screening was carried out using standard methods [25].

2.4 GC-MS Analysis

The extract was separated by gas chromatography by means of a Shimadzu (2010) model; GC was fixed with AB-Wax column. As a transporter gas helium was used. 0.1ml test sample was inserted in injector in splitless form. Detection of compounds was done by mass spectrophotometer. Chemical compounds separated from *S. viminalis* extract were confirmed by using Wiley spectral search program. The mass spectrum was detected in 40 min.

2.5 Strains and Growth Media

Different bacterial species *Escherichia coli* SN 1224 *(Gram -ve), Salmonella typhi* SN 0464 *(Gram –ve) and Staphylococcus aureus* SN 1175 *(Gram +ve)* were collected from Holy Family Hospital, New Delhi, India. These strains were maintained on 2% nutrient agar slants and were sub-cultured twice prior to testing, to ensure viability and purity. For all experimental studies bacterial cells were maintained on nutrient agar medium at -4°c [26].

2.6 MIC and Disc Diffusion Study

MIC of the test extract against different bacterial species was obtained as reported earlier [27]. Filter disc assay was performed by means of Kirby-Bauer modified disc dispersion technique. At -4°C strains were stored before they were used. Cells were grown at 37°C in nutrient agar and passaged twice on solid agar to achieve a lawn of confluent growth. Stock solutions of the test extract were prepared in 1% petroleum ether. Paper discs impregnated with different extract concentrations were poisoned on each plate. Paper disc dipped in 1% petroleum ether was positioned in center of disc that worked as solvent control. At 37°C for 48hrs plates were incubated. Diameter of the zone of inhibition was noted (in mm) after 2 days.

2.7 Growth Curve Studies

This experiment carried 10^6 cells {optical density A_{595}=0.1} of different bacterial strains which were cultured in presence of oxygen in programmed shaker maintained at 30°C until immobile (stationary) growth state was attained. Growth was followed at 595nm by applying spectrophotometer technique, which showed turbidness. The culture was added with the different concentrations of test extract. Growth phase of the cells alone and with inhibitor was performed. Each concentration was noted against visual concentration in time (hrs). Growth rate is exponential when optical density is compared against time duration.

3. Results and Discussion

3.1 Phytochemical & GC-MS analysis

Phytochemical screening of *S. viminalis* extract revealed that the petroleum ether leaf extracts contain glycosides, phenols, alkaloids, terpenoids, and flavonoids, except steroids, tannins & anthraquinones (Table 1).

Table 1: Preliminary phytochemical screening of petroleum ether leaf extracts of *S. viminalis*

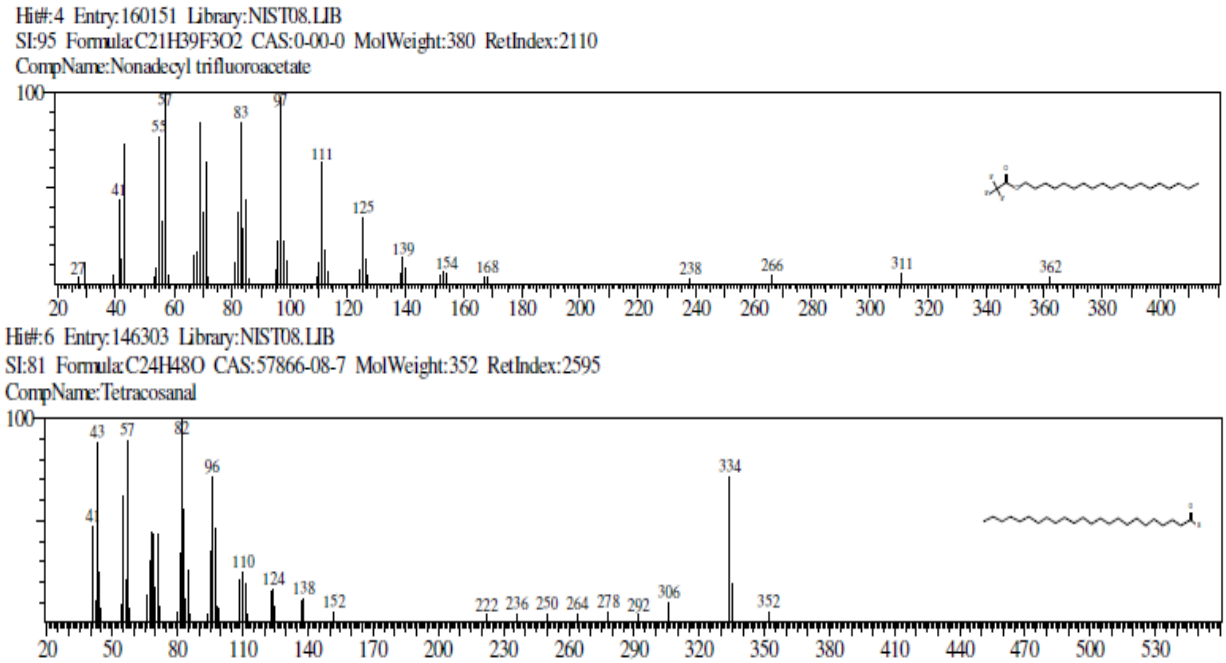
Constituents	Observation
Glycosides	Present
Phenols	Present
Alkaloids	Present
Terpenoids	Present
Flavonoids	Present
Steroids	Absent
Tannins	Absent
Anthraquinones	Absent

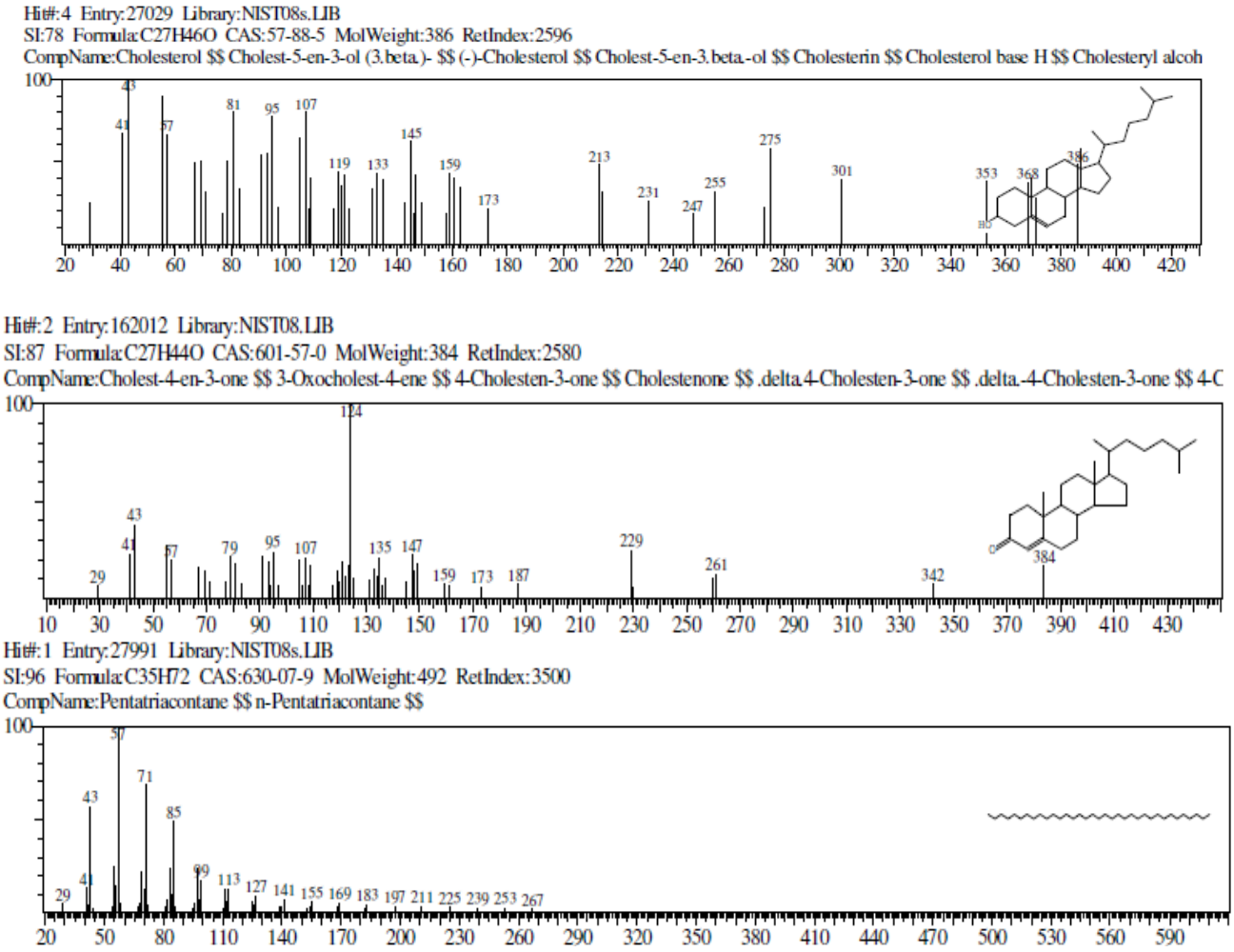
Results pertaining to GC-MS analysis lead to the identification of 47 different phytocompounds from GC fractions of the petroleum ether leaf extracts of *S. viminalis.* These phytocompounds were identified through mass spectrometry attached with GC (Table 2). The interpretation and nomenclature of phytocompounds is based on the molecular formula, molecular weight, retention time and percentage of presence. Till date no reports exist on the GC-MS analysis of petroleum ether leaf extracts of *S. viminalis.*

Table 2: Phytocompounds present in the Petroleum ether leaf extracts of *S. viminalis* using GC-MS analysis

S. No.	*Name of Compound*	*Molecular Formula*	*Molecular Weight*	*Retention Time*	*% of Presence*
1	**Nonadecyl trifluoroacetate**	**$C_{21}H_{39}F_3O_2$**	**380**	**25.61**	**10.72**
2	**Tetracosanal**	**$C_{24}H_{48}O$**	**352**	**25.13**	**9.01**
3	**Cholesterol**	**$C_{27}H_{46}O$**	**386**	**30.04**	**8.11**
4	**Cholest-4-en-3-one**	**$C_{27}H_{44}O$**	**384**	**32.03**	**8.02**
5	**Pentatriacontane**	**$C_{35}H_{72}$**	**492**	**23.63**	**5.41**
6	Methyl Commate D	$C_{31}H_{50}O_4$	486	30.94	3.62
7	2-Pyrrolidinone, 1-methyl-	C_5H_9NO	99	3.53	3.54
8	Heptacosan-1-ol	$C_{27}H_{56}O$	396	27.26	3.09
9	Palmitaldehyde	$C_{16}H_{32}O$	240	22.59	2.94
10	Methyl octacosanoate	$C_{29}H_{58}O_2$	438	27.52	2.64
11	2-Heptadecanone	$C_{17}H_{34}O$	254	27.43	2.35
12	Stearic acid ethyl ester	$C_{20}H_{40}O_2$	312	28.13	2.33
13	Ethyl pentacontanoate	$C_{52}H_{104}O_2$	760	24.73	1.92
14	1-Octacosanol	$C_{28}H_{58}O$	410	21.88	1.77
15	Palmitic acid, ethyl ester $$	$C_{18}H_{36}O_2$	284	26.33	1.75
16	Erythrodiol	$C_{30}H_{50}O_2$	442	30.28	1.62
17	Pentadecanal-	$C_{15}H_{30}O$	226	26.72	1.56
18	cis-1-Chloro-9-octadecene	$C_{18}H_{35}Cl$	286	28.66	1.52
19	Stearyl aldehyde	$C_{18}H_{36}O$	268	17.66	1.41
20	Cycloartane-3.beta.,25-diol	$C_{30}H_{52}O_2$	444	31.75	1.37
21	Vitamin E	$C_{29}H_{50}O_2$	430	27.74	1.32
22	Methyl tricosanoate	$C_{24}H_{48}O_2$	368	25.84	1.31
23	2-Pentacosanone	$C_{25}H_{50}O$	366	29.56	1.22
24	Methyl melissate	$C_{31}H_{62}O_2$	466	29.68	1.14
25	Tetratetracontane	$C_{44}H_{90}$	618	20.49	1.13
26	Docosyl heptafluorobutyrate	$C_{26}H_{45}F_7O_2$	522	29.36	1.12
27	5.alpha.-Stigmastane-3,6-dione	$C_{29}H_{48}O_2$	428	34.92	1.03
28	Methyl heneicosanoate	$C_{22}H_{44}O_2$	340	24.06	1.01
29	Triacontanoic acid	$C_{30}H_{60}O_2$	452	33.62	0.94
30	Isomenthol	$C_{10}H_{20}O$	156	16.73	0.91
31	Lignoceryl alcohol	$C_{24}H_{50}O$	354	19.27	0.8
32	Arachidic alcohol	$C_{20}H_{42}O$	298	17.4	0.75
33	1-Heptatriacontanol	$C_{37}H_{76}O$	536	19.71	0.75
34	2-Heptacosanone	$C_{27}H_{54}O$	394	25.74	0.61
35	gamma.-Tocopherol	$C_{28}H_{48}O_2$	416	26.97	0.61
36	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	17.21	0.6
37	Methyl isomyristate	$C_{15}H_{30}O_2$	242	14.9	0.35
38	Oxacyclotetradecan-2-one	$C_{13}H_{24}O_2$	212	15.23	0.32
39	Oleic acid, methyl ester	$C_{19}H_{36}O_2$	296	16.61	0.29
40	Heptadecanoate	$C_{19}H_{38}O_2$	298	15.57	0.25
41	Methyl heptacosanoate	$C_{28}H_{56}O_2$	424	26.64	0.25
42	3-Ethyloctane	$C_{10}H_{22}$	142	3.2	0.23
43	Dodecane	$C_{12}H_{26}$	170	5.76	0.23
44	Isocyanic acid, octadecyl ester	$C_{19}H_{37}NO$	295	18.99	0.2
45	2-Propyl-1-pentanol	$C_8H_{18}O$	130	3.29	0.19
46	Butyl phthalate	$C_{16}H_{22}O_4$	278	15.36	0.18
47	Heneicosane	$C_{21}H_{44}$	296	18.33	0.18

Five compounds were found in the major concentration. Fragmentation pattern of these five compounds is given below.





4. Biological Investigation

4.1 MIC & Filter Disc Assay

Petroleum ether leaf extracts of *S. viminalis* displayed considerable MIC's levelling from 800 to 1600 µg/ml for *Escherichia coli* SN 1224 *(Gram -ve), Salmonella typhi* SN 0464 *(Gram –ve) and Staphylococcus aureus SN 1175 (Gram +ve)* Table 3. Antibacterial activity of Petroleum ether leaf extracts of *S. viminalis* at 3 dissimilar concentrations viz, 4 mg/ml, 8 mg/ml & 12 mg/ml

Table 3: MIC screening data of Petroleum ether leaf extracts of *S. viminalis* (µg/ml) against different bacterial isolates

Bacterial Species	*MIC of Petroleum ether leaf extracts of S. viminalis (µg/ml)*
SN 1224 *Escherichia coli (Gram -ve)*	800
SN 0464 *Salmonella typhi (Gram -ve)*	1000
SN 1175 *Staphylococcus aureus (Gram +ve)*	1600

was studied against *Escherichia coli* SN 1224 *(Gram -ve), Salmonella typhi* SN 0464 *(Gram –ve)* and *Staphylococcus aureus SN 1175 (Gram +ve).* Results are summarised in (Table 4) and revealed in figure 1. Observations of Petroleum ether leaf extracts of *S. viminalis* against all the bacterial isolates has revealed significant antibacterial activity. At 4 mg/ml, extract exhibited minimum inhibitory activity against all the bacterial isolates.

Against control disc (1% DMSO) there was found no change, hence 1% DMSO using as solvent has no effect on the tested bacterial strains. On the basis of results observed, we can conclude that Petroleum ether leaf extracts of *S. viminalis* exhibited significant anti-bacterial activity. Relationship between *in vitro* anti-bacterial activities of the extract at dissimilar concentrations & standard drug has been shown in Figure 2.

The percentage of inhibition varied with the concentration of the plant extract. Against *Salmonella typhi* SN 0464 *(Gram –ve)* maximum zone of inhibition (Z.O.I) i.e., 12.80mm was measured when treated with 12mg dose of plant extract, 11.40mm was measured for *Salmonella typhi* SN 0464 *(Gram –ve)* and *10.15mm* for *Staphylococcus aureus SN 1175 (Gram +ve)* when treated with the same concentration of the extract.

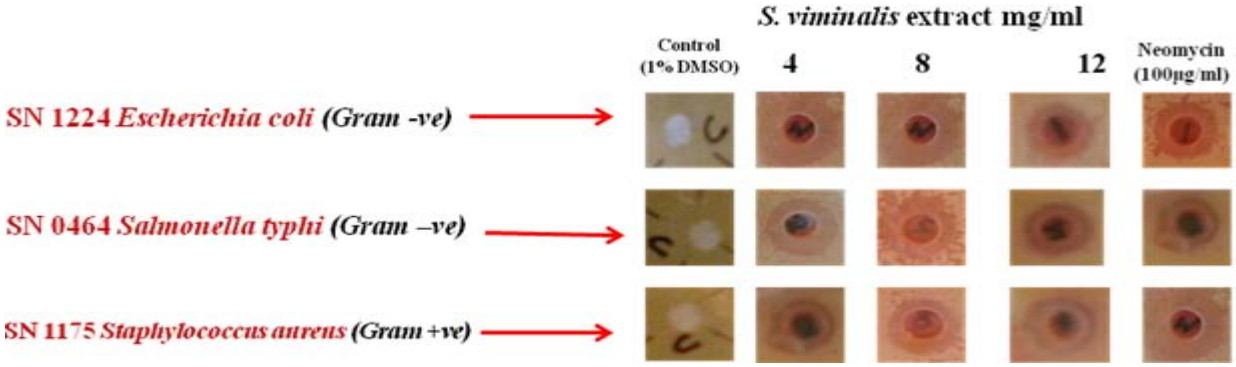


Figure 1: Photographs obtained in filter disc assay of the test extract against *Escherichia coli* SN 1224 *(Gram -ve)*, *Salmonella typhi* SN 0464 *(Gram –ve)* and *Staphylococcus aureus SN 1175 (Gram +ve).*

Table 4: Anti-bacterial activity screening data for different test extract concentrations and Neomycin

Zone of Inhibition (mm)			
Test extract	*SN 1224 Escherichia coli (Gram -ve)*	*SN 0464 Salmonella typhi (Gram –ve)*	*SN 1175 Staphylococcus aureus (Gram +ve)*
4mg/ml	3.31±0.11	5.65±0.18	4.11±0.50
8mg/ml	8.65±0.49	9.65±0.40	10.23±0.35
12mg/ml	10.15±0.81	**12.80±0.75**	11.40±0.72
Neomycin [a] (100µg/ml)	14.16±0.75	15.59±0.57	15.59±0.49
Control [b] (1% DMSO)	-	-	-

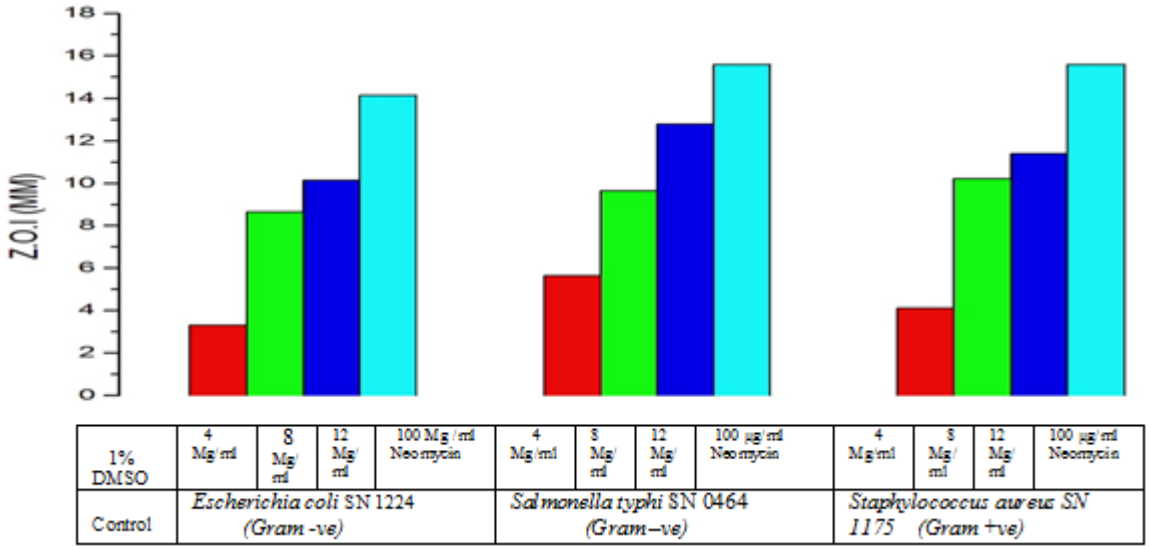
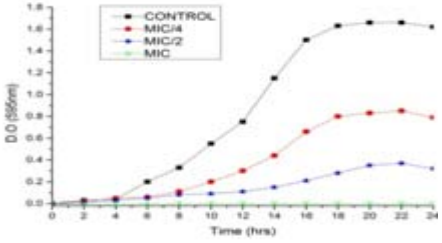


Figure 2: Bar diagram showing comparison between anti-bacterial activities of Petroleum ether leaf extracts of *S. viminalis* at different concentrations and standard anti-bacterial drug against **(a)** *Escherichia coli* SN 1224 *(Gram -ve)* **(b)** *Salmonella typhi* SN 0464 *(Gram –ve)* **(c)** *Staphylococcus aureus SN 1175 (Gram +ve)*

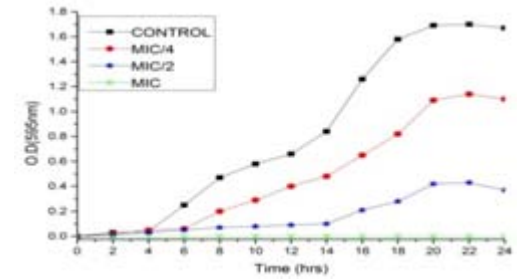
4.2 Growth Curve Studies

(Turbidness Measurement):

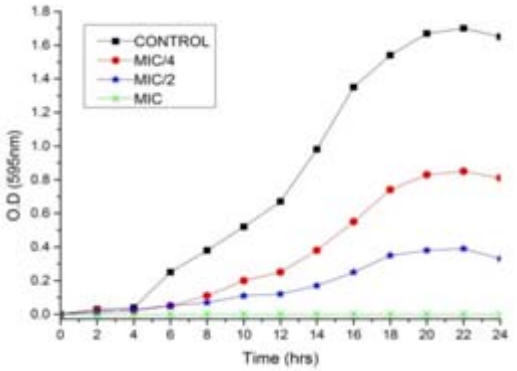
Growth curve of the *bacterial* species was investigated at different concentrations of Petroleum ether leaf extracts of *S. viminalis.* Figure 3a, 3b & 3c with dissimilar concentrations of Petroleum ether leaf extracts of *S. viminalis* showed different effect on growth pattern of *Escherichia coli* SN 1224 *(Gram -ve)*, *Salmonella typhi* SN 0464 *(Gram –ve)* and *Staphylococcus aureus SN 1175 (Gram +ve)*. 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 it showed normal curve. Increase in the concentration of the extract showed decrease in growth with concealed and deferred exponential phase in comparison to control. Initially this showed lag phase, then exponential phase and at last a stationary phase. No growth was seen which is shown by smooth line at minimum inhibitory concentration values.



(a) *Escherichia coli* SN 1224 *(Gram -ve)*



(b) *Salmonella typhi* SN 0464 *(Gram –ve)*



(c) *Staphylococcus aureus SN 1175 (Gram +ve)*

Figure 3: Effect of different concentrations of Petroleum ether leaf extracts of *S. viminalis* on growth of different bacterial species. Growth curve pattern against absorbance at 595nm (hrs) shows complete inhibition of growth at MIC values **(a)** Against *Escherichia coli* SN 1224 *(Gram -ve)* **(b)** Against *Salmonella typhi* SN 0464 *(Gram –ve)* **(c)** Against *Staphylococcus aureus SN 1175 (Gram +ve)*

5. Discussion

The potential for obtaining antimicrobial agents from medicinal plants seems satisfying, as it will guide to the enhancement of natural medicine to be used against different pathogens. Our findings provide an idea for intensifying the efficacy of plant active principals as anti-bacterial agents. According to the results Petroleum ether leaf extracts of *S. viminalis* exhibited antibacterial activity, shown by filter disc assay & growth curve study against *Escherichia coli* SN 1224 *(Gram –ve)*, *Salmonella typhi* SN 0464 *(Gram –ve)*, *Staphylococcus aureus SN 1175 (Gram +ve*. As a function of various concentrations growth kinetic studies also pursue the similar trend while as tested cells of MIC/4 shows dejected expansion curves with clearly distinguished growth phases. MIC/2 treated cells revealed concealed and late exponential growth phase. Finally at MIC value S shaped growth curve reduced to smooth (flat) line viewing nearly complete death of cell growth (Fig. 3). Filter disc assay solid media revealed efficient inhibition of growth of different *bacterial* isolates with the test extract and was found to enhance in absorption dependent approach Figure 1.

6. Conclusion

Petroleum ether leaf extracts of *S. viminalis* has revealed potent anti-bacterial effect in both solid & liquid medium. This research work is a supplementary attempt for development of new therapeutic agents which is anti-bacterial, less poisonous & helps in prevention of drug resistance. Additional examination and testing needs to be done which is very necessary, that may help to make possible applications of this extract in future as anti-bacterial agent.

7. Acknowledgement

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Author Profile

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