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# PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF *SALVIA SPLENDENS* SELLO.

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*The phytochemical investigation of antimicrobial agents from locally occuring Salvia splendens Sello. was carried out. Three common solvents i.e. petroleum ether (40-60°), chloroform and methanol were successively used for the extraction of antimicrobial principles from its various parts. Three major compounds (namely $C_1$, $C_2$ and $C_3$) were isolated and purified from the active methanol extract of roots by column and thin layer chromatography. Gram (+) bacteria (Bacillus pumilus, B. subtilis and Staphylococcus aureus), gram (–) bacteria (Proteus vulgaris, Escherichia coli and Pseudomonas aeruginosa) and a fungus (Candida albicans) were used to measure the zones of inhibition of these compounds by a known method. One of the compound $C_1$ gave larger zones than the other two isolated compounds ($C_2$ and $C_3$). An attempt was made to identify the nature of antimicrobial compounds by available spectral means. A possible structure- activity relationship of the potent antimicrobial compounds was discussed.*

**Keywords** : *Antimicrobial activity; Zones of inhibition; Salvia splendens*

## Introduction

*Salvia splendens* Sello. (Lamiaceae) is an annual ornamental herb with typical aromatic smell. It is a Brazilian plant with red flowers. In Pakistan, it is found as wild and also cultivated in the plains of Punjab, Sind and Baluchistan (1-4). A substantial research work on the isolation, characterization and the pharmacological action of various phytochemical compounds from a large number of Lamiaceae members have been done in the past, but only a few antimicrobial principles have been reported (5-9). The plants of this family including different species of *Salvia* are valuable sources of essential oils which are mostly used as flavouring agents in cosmetic and perfumery industry and also in medicines (1,3-6).

Most of the *Salvia* species are carminative and stimulant for allaying nausea, sickness, vomiting and fever (1-3). The oil extracted from the leaves of *S.splendens* is widely used by the local people against topical infection and also against mosquito's bites (1,4).

The principal chemical components of the essential oils, which are present in the glandular hairs of the different *Salvia* species had previously been determined (5-10), while the chemical constituents from the other parts of different species of genus *Salvia* had also been isolated and characterized in the past (5,11-19).The reports on the antimicrobial activities of the genus *Salvia* have been lacking in the literature.

The present work was conducted to isolate and purify the active principle from various parts of *S. Splendens* by successive solvent extraction and to evaluate its antimicrobial potential. This will posibbly lead to the structure-activity relationship of potent antibacterial/antifungal compounds in this particular species.

### Materials and Methods

*General*

All the solvents and chemicals used in this study were of analytical grade. Saturated lead acetate solution was prepared by dissolving 100 g of lead acetate in 160 ml of distilled water. Sodium sulphate solution was prepared by dissolving 6.3 g of anhydrous sodium sulphate in 100 ml of distilled water.

### Plant Materials

*Salvia splendens* Sello. plants were collected from the botanical garden, Government College,

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Lahore and from local places around Jinnah Garden, Lahore during March/April 1996. These were authenticated by Dr. Zaheer-ur-Din Khan, Taxonomist, Department of Botany, Goverment College, Lahore. The voucher specimen was deposited in the Herbarium of Pharmacognosy Section, Department of Pharmacy, University of the Punjab, Lahore.

Leaves, stems and roots of the plants were separated and dried under the shade at room temperature. The dried plants were pulverized to a fine powder and stored in amber coloured bottles.

*Test Organisms*

The pure cultures of *Bacillus pumilus* NCTC 8241, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 29737 (Gram+); *Proteus vulgaris, Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 25619 (Gram-); *Candida albicans* (Fungus) were obtained from Drug Testing Lahoratories, Lahore. All the bacterial and fungus strains were maintained at 37°C and 25°C on Nutrient agar and Sabouraud's dextrose agar media respectively.

*Spectral Analysis*

UV spectra of the isolated compounds were recorded on Hitachi-270-30 spectrophotometer using methanol as a solvent and IR spectra were measured on Pye-Unicam SP-8-400 spectrophotometer using thin film on NaCl disc.

*Solvent Extraction*

Powdered, leaves (500 g), stem (500 g) and roots (500 g) were extracted successively in petroleum ether (40-60°), chloroform and methanol by using 1.5 litre of each solvent for soaking the roots and stems and 2 litre for soaking the leaves. Maceration was carried out in each solvent for three days at room temperature (27±2.5°C). The solvent of each extracted material was removed under reduced pressure and the residues were weighed. The yield of crude residues are given in Table 1.

*Removal of Chlorophyll*

Each of the solvent extracted material from leaves and stems was treated with excess of saturated lead acetate solution in a beaker and stirred. In case of petroleum ether and chloroform extracts, the solvent layers were removed and treated with excess of sodium sulphate solution, while in case of methanol extract the whole mixture was treated with excess of sodium sulphate solution. The precipitated chlorophyll was removed by filtration and each solvent was evaporated under reduced pressure to obtain chlorophyll free residues(20).

*Thin Layer Chromatography*

The silica gel G-60 (E. Merck) thin layer plates (0.25 mm thick) were prepared with the help of moving spreader (Dosga applicator). For a comparative TLC analysis, following solvent systems in different ratios, i.e. 40:60, 50:50, 60:40 (v/v) were used respectively: I-Benzene:methanol, II-Chloroform:methanol, III-Ethyl acetate: methanol, IV- Acetic acid:methanol V-Methanol:HCl. Visualization of the chromatograms were achieved by UV light, iodine and 5% $H_2SO_4$ in methanol.

*Column Chromatography*

The methanol extract of roots of *S. Splendens* (15 g) was subjected to fractionation on silica gel G-60 (70-230 mesh) column (300 g). Ethyl acetate was used for packing the column. The column was eluted first with ethyl acetate, then polarity of the system was raised by increasing the quantity of methanol in ethyl acetate. 20 ml fractions were collected and the fractions having similar compounds were pooled together after monitoring with thin layer chromatography. The isolated compounds were further subjected to spectral analysis, phytochemical screening and antimicrobial study.

*Phytochemical Screening*

Pooled column fractions were tested for the presence of flavonoids, phenols, tannins, terpenes and sterols according to the method described by Farnsworth(21).

*Antimicrobial Activity*

The crude extracts and purified compounds were studied for antimicrobial activity. The bacterial and fungal suspensions were prepared by suspending a loopful of the pure culture (24 hours old bacteria and 72 hours old fungus) in 10 ml sterile distilled water. One ml of bacterial suspensions were separately mixed with 14 ml of sterile molten N.A. medium in different sterile petri dishes (already labeled with bacteria and compound's name under study). The fungus suspension (1ml) was mixed with 14 ml of sterile Sabourand's dextrose agar medium in different petri dishes. The media in all petri dishes were then allowed to solidify at room temperature. Filter paper discs of 6 mm in diameter were sterilized and soaked into the solutions of crude extracts and purified compounds. The filter paper discs (solvent dried) were placed in the petri dishes at their labeled positions. Petri dishes with bacterial inoculation were incubated at 37°C for 24 hours, while petri dishes with fungal inoculation were incubated at 25°C for 72 hours. At the

end of the incubation periods, inhibition zones were measured with the help of a vernier calliper.

The mean diameter of inhibition zones, against the microorganisms produced by six replicates of crude extract and isolated compounds, were calculated along with their effective ranges.

## Results and Discussion

*Salvia splendens* Sello., plants (Laminaceae) when collected from the botanical garden and from the local waste places, had variable appearance. During collection in different seasons from diverse localities, it was observed that the local climatic conditions probably have a great effect on the appearance of the plants. On the basis of such ecological differences, it was postulated that the plant as a whole or its parts might contain different types of secondary metabolites (1-4). It was observed by many workers that the oil extracted from this species, was often used by the Indian people as a folk medicine for topical infection, particularly in toothache and also against mosquito bites (1-4,11). Moreover, the wild plants of this species are also commonly found as weeds in the fields of other economical and ornamental plants and cause a lot of damage to them. Taking into account these observations, the present phytochemical investigation of antimicrobial agents from this local plant was carried out. The exact nature and identity of such antimicrobial principle present in various parts of this species if any, has not previously been investigated.

Leaves, stems, roots, and flowers of this plant were available during the collecting season. First three parts were gathered in larger amounts than others, while the amount of flowers were insufficient to be manipulated further. For this reason, leaves, stems and roots of this species were subsequently explored for the isolation and identification of their active antimicrobial phytochemical compound/s.

For the isolation and purification of phytochemical compounds, a broad solvent extraction was carried out. For this purpose, a range of both non-polar and polar solvents i.e. petroleum ether (40-60°), chloroform and methanol were used. The dried powdered leaves, stems and roots of *S. splendens* were thus subjected to successive extraction in these three solvents under the laboratory conditions.

Solvent extracted material contained a large amount of chlorophyll pigments which were assumed to interfere the isolation of the phytochemical compounds present in these extracts. The solvent extracted residues were thus subjected to the removal of chlorophyll using a routine process(20). The results of the broad solvent extraction in the form of percentage yield of each part in the solvent used, have been outlined in Table 1. The results indicated that all the three parts (leaves, stem and roots) of this plant contained a larger proportion of methanol soluble polar compounds than both the petroleum ether and chloroform soluble non-polar components.

The solvent extracted materials from three plant parts were then subjected to the comparative TLC analysis using different solvent systems. The main

Table 1. Yield of the extracted materials obtained from different parts of S. splendens by different solvents

| Plant Parts | Solvent Extracts | Amount (g) | Percentage |
|---|---|---|---|
| Leaves | Petroleum ether Extract | 5.50 | 1.1 |
| | Chloroform Extract | 6.43 | 1.28 |
| | Methanol Extract | 9.40 | 1.9 |
| Stems | Petroleum ether Extract | 3.41 | 0.68 |
| | Chloroform Extract | 2.41 | 0.48 |
| | Methanol Extract | 8.0 | 1.60 |
| Roots | Petroleum ether Extract | 1.44 | 0.29 |
| | Chloroform Extract | 1.09 | 0.22 |
| | Methanol Extract | 15.50 | 3.10 |

Table 2. Comparative thin layer chromatographic results of the methanol extract of different plant parts of *S. splendens*

| Solvent system | Ratio | PLANT PART USED | | | | | |
|---|---|---|---|---|---|---|---|
| | | LEAVES | | STEMS | | ROOTS | |
| | | No of com. | hRf values | No of com | hRf values | No of com | hRf values |
| Benz./MeOH | 40:60 | 4 | 67, 78, 85, 92 | 4 | 10, 17, 64, 75 | 0 | 0 |
| | 50:50 | 5 | 49, 56, 59, 72, 80 | 3 | 13, 34, 65 | 0 | 0 |
| | 60:40 | 6 | 17, 65, 72, 89, 93, 96 | 2 | 52, 59 | 0 | 0 |
| $ChCl_3$/MeOH | 40:60 | 2 | 56, 91 | 3 | 11, 56, 88 | 1 | 13 |
| | 50:50 | 3 | 14, 46, 82 | 2 | 32, 65 | 1 | 23 |
| | 60:40 | 4 | 29, 47, 65, 91 | 1 | 14 | 1 | 14 |
| Ethyl acetate/ MeOH | 40:60 | 5 | 35, 50, 64, 78, 89 | 6 | 7, 16, 28, 46, 75, 89 | 6 | 14,23,35,60,67,85 |
| | 50:50 | 3 | 13, 78, 88 | 2 | 9, 68 | 3 | 13, 78, 84 |
| | 60:40 | 4 | 64, 67, 70, 89 | 4 | 67, 70, 76, 85 | 4 | 16, 23, 64, 87 |
| Acetic acid/ MeOH | 40:60 | 2 | 78, 92 | 1 | 79 | 1 | 89 |
| | 50:50 | 2 | 76,86 | 1 | 73 | 1 | 86 |
| | 60:40 | 2 | 75,85 | 1 | 75 | 1 | 82 |
| MeOH/HCl | 40:60 | 1 | 79 | 1 | 78 | 1 | 87 |
| | 50:50 | 1 | 73 | 1 | 73 | 1 | 85 |
| | 60:40 | 1 | 75 | 1 | 72 | 1 | 87 |

Table 3. Physica-chemical characteristics of purified compounds isolated from the methanol extract of roots of *S. splendens*

| No. | Characteristics | Compound $C_1$ | Compound $C_2$ | Compound $C_3$ |
|---|---|---|---|---|
| 1 | Colour | Brownish yellow | Yellow | Light yellow |
| 2 | Physical | Thick viscous | Thick viscous | Thick viscous |
| 3 | Fluorescence | Pink | Pink | Pink |
| 4 | Reaction with iodine | Yellow | Yellow | Yellow |
| 5 | Reaction with o-dianisidine* | Red (Ketonic group) | Red (Ketonic group) | Red (Ketonic group) |
| 6 | Reaction with nitroprusside (sodium)/ NaOH (Legal Test) | Violet (Ketonic group) | Pink (Aldehydic group) | ------------- |
| 7 | Reaction with hydroxyamine/ferric chloride** | | Brown (Phenolic group) | Dark brown (Tannin) |
| 8 | Reaction with $SbCl_3$ | | -------------- | ------------- |
| 9 | UV absorption (λmax) nm | 232, 275 | 230, 275, 330 | 210, 275 |
| 10 | IR absorption ($cm^{-1}$) | 3500(s), 2950 (s), 1740 (w), 1475 (s), 1400 (m), 1275 (w) 725 (w) | 3500 (s), 2950 (s), 1630 (w), 1478 (s), 1390 (m), 739 (m) | 3500 (s), 2950 (s), 1470 (s), 1390 (m) 725 (w) |

(*) Staturated solution of o-dianisidine in acetic acid

(**) Solution I : A mixture (in 1:2 ratio) of aqueous ethanolic solution of hydroxyl ammonium chloride (2 g in 20 ml w/v) + aqueous ethanolic solution of KOH (2 g in 25ml w/v).

Solution II : 1 g $FeCl_3$ in 2 ml 36% HCl w/v + 20 ml $(CH_3)_2O$ Solutions were mixed prior to usage

purpose of these analysis was to have a conviction of the total number and chromatographic behaviour of the compounds present in each extract. The results of these analysis have been outlined in Table 2. The best solvent system which resolved the mixture of methanolic extracts of leaves into six major components seemed to be benzene/methanol (60:40). The methanolic extract of stem was segregated into six camponents by the solvent system-ethyl acetate/methanol (40:60). Similarly methanol extract of root was best segregated into six components by ethyl acetate/methanol in 30:70 ratio (Table 2).

Preliminary antimicrobial tests were performed with all three types of solvent extracts. The results indicated that the methanolic extract gave a well defined

response, while other extracts gave no reaction. Since methanolic extract of the roots of *S. splendens* appeared comparatively more active and also was obtained in larger amounts than the other active extracts, it was further subjected to column chromatographic analysis to isolate the active compound/s using an increasing quantity of methanol in ethyl acetate. The elution process was monitored by thin layers. Eight pooled fractions were obtained, based on the thin layer chromatographic analysis. Out of the eight pooled column fractions, the second, seventh and eighth column fractions were active and produced well marked zones of inhibitions against the types of microorganisms used. Three compounds were isolated from second, seventh and eighth active pooled column fractions of the methanol extract of roots of *S. splendens*. They were named as $C_1$, $C_2$ and $C_3$. The physico-chemical characteristics of these purified compounds have been outlined in Table 3.

The compound $\mathbf{C_1}$ was isolated and purified from second pooled column fraction. It was a yellowish brown semisolid compound and chromatographically pure. Thin layer chromatography of this compound indicated only one major spot, when a number of solvent systems (Table 2) were used It gave yellow colour when iodine vapouurs were used as a detecting agent, under daylight conditions. Under UV light, it gave pink spots on purple background. The available spectral evidence showed that the compound $C_1$ was probably a conjugated diene containing methyl, ketonic or carboxylic acid group along with some –OH groups (22,23).

The compound $\mathbf{C_2}$ was isolated from seventh pooled column fraction of methanol extract of roots. It exhibited a single spot after developing on silica gel plates tlc (Table 2) iodine vapours and UV light were used. For detection spectral evidence showed the presence of methyl and ketonic or carboxylic acid group with some free –OH groups probably due to the presence of some alcohols or phenols (22,23).

The third compound $\mathbf{C_3}$ was isolated and purified from the eighth pooled column fraction. This was a yellow brown thick viscous compound and was chromatographically pure. Thin layer chromatography of this compound indicated only one major spot, when a number of solvent systems (Table 2) were used. It gave yellow colours when iodine vapours were used as a detecting agent, under ordinary light conditions. Under UV light, it gave pink spot or purple back ground. The available spectral evidence indicated that the compound $C_3$ was also probably a conjugated diene containing methyl, ketonic or some alkane type of hydrocarbon along with some –OH groups (22,23).

Antimicrobial activity of methanol extract of roots and purified compounds ($C_1$, $C_2$ and $C_3$) were tested againts the available microorganism. The results indicated that all the three types of microorganisms were markedly effected by the crude methanol extract and their purified compound $C_1$. The compound $C_2$ showed an inhibitory effect only on the four microorganisms including B. *stubtilis, S. aureus, P. Aeruginisa* and *C. Albicans*. The compuond $C_3$ exhibited an inhibitory effect on *B. subtilis, C. aureus, P. Vulgaris* and *C. albicans* (Table 4). The results further showed that the compound $C_1$ was a more potent antimicrobial agent against both types of bacteria and fungus than other compounds (Table 4). Since these compounds contained methyl, ketonic or carboxylic acid group along with conjugated diene system in their molecules, probably they penetrate easily through the bacterial cell and retard their growth

Table 4. Antimicrobial activity of crude and purified compounds of methanol extract of roots of *S. splendens*

| MICROORGANISMS | ZONES OF INHIBITION (mm)* | | | |
|---|---|---|---|---|
| | Crude Extract | Compound $C_1$ | Compound $C_2$ | Compound $C_3$ |
| Gram + bacteria | | | | |
| Bacillus pumilus | 36.55±2.04 | 22.35±4.31 | --------------- | ------------- |
| Bacillus substilis | 20.00±1.03 | 1.00±2.13 | 6.00±1.25 | 8.50±0.41 |
| Staphylococcus aureus | 38.65±1.67 | 5.50±3.51 | 9.00±1.04 | 26.40±3.7 |
| Gram- bacteria | | | | |
| Proteus vulgaris | 32.45±0.25 | 12.50±2.50 | ----------- | 22.25±3.4 |
| Escherichia coli | 21.25±1.01 | 10.00±1.50 | ----------- | --------- |
| Pseudomonas aeruginosa | 22.00±2.0 | 10.00±1.66 | 13.00±2.2 | |
| Fungus | | | | |
| Candida albicans | 25.35±4.80 | ------------ | 15.25±2.3 | 35.55±5.41 |

or completely kill them (8,9,24).

It could be concluded that a detailed characterization of these compounds is necessary so that a structure-activity releationship in terms of antimicrobial activity could be developed.

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