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PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF *SALVIA SPLENDENS* SELLO

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ABSTRACT:

The phytochemical investigation of antimicrobial agents from locally occurring *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 positive bacteria *(Bacillus pumilus, B. subtilis* and *Staphylococcus aureus),* Gram negative bacteria *(Proteus vulgaris, Escherichia coil* and *Pseudomonas aeruginosa)* and a fungus *(Candida albicans)* was used to measure the zones of inhibition of these compounds by 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.

INTRODUCTION

Salvia splendens Sello. (Lamiaceae) is an annual ornamental herb with typical aromatic smell (Evans 1989; Kirtikar and Basu 1987; Pandy 1988; Pandy 1989). 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 (Evans 1989; Kirtikar and Basu 1987; Pandy 1988; Pandy 1989). 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 from them (Asthana et al., 1986; Colin 1990; Dikshit and Hussain 1984; Li et al., 1991 and Maita et al., 1985). 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 (Colin 1990; Evans 1989; Li et al., 1991; Kirtikhar and Basu 1987; Pandy 1988).

Most of the Salvia species are carminative and stimulant for allaying nausea, sickness, vomiting and fever (Evans 1989; Kirtikar and Basu 1987; Pandy 1989). 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 (Kirtikar and Basu 1987; Pandy 1988).

The principal chemical components of the essential oils, which are present in the glandular hairs of 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 (Elnir et al. 1991; Falk et al. 1990; Gonzalez et al. 1990; Li et al. 1991; Lu et al. 1991; Sur 1991; Tanker et al. 1986; Tomas-Bakberan et al. 1988; Ulubelen and Topcu 1991; Yasumasa et al. 1991). The reports on the antimicrobial activities of different species of genus *Salvia* have been lacking in the literature (Diaz and Quevedo-Sarmiento 1988; Gergis et al. 1991).

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 potentials. This will possibly 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 100g of lead acetate in 160m1 of distilled water. Sodium sulphate solution was prepared by dissolving 6.3g of anhydrous sodium sulphate in 100ml of distilled water.

Plant Materials:

Salvia splendens Sello, plants were collected from the Botanical garden, Government College Lahore and from local places around Jinnah Garden Lahore during March/April 1997. These were authenticated by Dr. Zaheer-Ud-Din Khan, Taxonomist, Department of Botany, Government 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 2937 (Gram positive); *Proteus vulgaris, Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 25619 (Gram negative); Candida *albicans* (Fungus) were obtained from Drug Testing Laboratories 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 spectro-photometer 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 (500g), stem (500g) 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 (Brain and Turner 1975). 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 of the solvent was evaporated under reduced pressure to obtain chlorophyll free residues (Brain and Turner 1975),

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 comparative TLC analysis, following solvent systems in different ratio, i.e. 40:60, 50:50, 60:40 (v/v) respectively were used: I-Benzene/methanol, II-Chloroform/methanol, III-Ethyl acetate/methanol, IV-Acetic acid/methanol, V-Methanol/HCI. Visualization of the chromatograms were achieved by UV light, with iodine and with 5% H_2SO4 in methanol.

Cabana Chromatography:

The methanol extract of roots of *S. splendens* (15g) was subjected to fractionation on silica gel Gm (70-230 mesh) column (300g). 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. 20ml 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 (1966).

Antimicrobial Activity:

The crude extracts and purified compounds were studied for antimicrobial activity. The bacterial and fungal suspensions were prepared by suspending a loop-full of the pure culture (24 hours old bacteria and 72 hours old fungus) in 10m1 sterile distilled water. One ml of bacterial suspensions were separately mixed with 14m1 of sterile molten. N.A. medium in different sterile petri dishes (already labeled with bacterial and compound's name under study). The fungus suspension (lml) was mixed with 14ml 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 6mm in diameter were made sterilized and Soaked in the solutions of crude extracts and purified compounds. The filter paper discs (solvent dried) were placed in the petri dishes at their respective 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 replicate of crude extract and isolated compounds, were calculated along with their effective ranges.

RESULTS AND DISCUSSION

Salvia spleldens Sello. plants (Laminaceae) when collected from the botanical garden and from the local waste places, appeared to have 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 it's parts might contain different types of secondary metabolities (Evans 1989; Kirtikar and Basu 1987; Pandy 1988; Pandy 1989). It was observed by many workers that the oil extracted from this species, is often used by the Indian people as a folk medicine for topical infection, particularly in toothache and also against mosquito bites (Evens 1989; Kirtikar and Basu 1987; Pandy 1989; Tanker et al. 1986). Moreover, the wild plants of this species are also commonly found as weeds in the fields of other economical and ornamental plants and causes a lot of damage to them. Taking into account these observations, the present phytochemical investigation of atimicrobial 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 flowers were insufficient amount 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 for three days under the laboratory conditions.

Solvent extracted materials 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 (Brain and Turner 1975). The result 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 less polar components (Table-1).

The solvent extracted materials from these three plants were then subjected to the comparative TLC analysis using different solvent systems. The main purpose of these analyses was to have a conviction of the total number and chromatographic behaviour of the compounds present in each extract. The results of these analyses have been outlined in Table-2. The best solvent system, which resolved the mixture of methanolic extract of leaves into six major components seemed to be benzene/methanol (60:40) (Table-2). The methanolic extract of stem was segregated into six components by the solvent system—ethyl acetate/methanol (40:60) (Table-2). Similarly methanol extract of root was best segregated into six components by ethyl acetate/methanol in 30:70 ratio (Table-2)

Preliminary antimicrobial test was performed with all three types of solvent extracts. The results indicate that the methanolic extract gave a well defined response, while other extracts gave no reaction. Since methanolic extract of the roots of *S. splendens* was active and was present in larger amount than 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 C_1** was isolated and purified from second pooled column fraction. It was a yellowish brown semi-solid compound and chromatographically pure. Thin layer chromatography of this compound indicated only one major, spot, when a number of solvent system (whose list is given in Table-2) were used. It gave yellow colour when iodine vapours were used as a detecting agent, under ordinary light conditions. Under UV light, it gave pinkish spots on purple back ground. Other physico-chemical characteristics have been outline in Table-3. The available spectral evidence showed that the compound CI was probably a conjugated diene containing methyl, ketonic or carboxylic acid group along with some-OH groups.

The **compound C_2** was isolated from seventh pooled column fraction of methanol extract of roots. It exhibited a single spot on silica gel thin layers under the influence of different solvent systems whose list has been outlined in Table-2 and were detected by iodine vapours and UV light. The available sectral evidence showed the presence of methyl and ketonic or carboxylic acid group with some free-OH groups probably of some alcohols or phenols.

The third **compound C_3** was isolated and purified from the eighth pooled column fraction. This was a yellowish brown thick viscous compound and was chromatographically pure. Thin layer chromatography of this compound indicated only one major spot, when a number of solvent system (whose list is given in Table-2) were used. It gave yellow colour when iodine vapours were used as a detecting agent, under ordinary light conditions. Under UV light, it gave pinkish spot on purple back ground. The available spectral evidences, 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.

Antimicrobial activity of methanol extract of roots and purified compounds (C_1, C_2 and C_3) were tested against the available microorganisms. Their effects were compared with the commercially available antibiotics under the same laboratory conditions. The results indicated that all the three types of microorganisms were markedly effected by the crude methanol extract and its purified compound C_1. The Compound C_2 showed an inhibitory effect only against the four microorganisms including *B. stubtilis, S. aureus, P. aeruginosa* and *C. albicans*. The compound C_3 exhibited an inhibitory effect on *S. subtilis, S. aureus, P. vulgaris* and *C.albicans* (Table-4). The results further showed that the compound C_1 is more potent antimicrobial agent against both types of bacteria and fungus than the other two compounds (Table-4). Since these compounds contained methyl, ketonic or carboxylic acid groups along with conjugated diene system in their molecules, probably easily penetrate through the bacterial cell membrane (Asthana et al. 1986; Dikshit and Hussain 1984; Gergis et at. 1991), either retarding their growth or completely killing them (Asthana et at. 1986; Dikshit and Hussain 1984; Gergis et al. 1991).

It could further be concluded that a detailed characterization of these compounds is necessary, so that a structure-activity relationship in terms of their antimicrobial activity could be established.

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

Plant Parts	Solvent Extracts	Amount (g)	% age
Leaves	Petroleum ether Extract	5.50	1.1
	Chloroform Extract	6.43	1.28
	Methanol Extract	9.40	1.09
Stems	Petroleum ether Extract	3.41	0.68
	Chloroform Extract	2.41	0.48
	Methanol Extract	8.00	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	Plants Part Used					
		Leaves		Stems		Root	
		No. of Corn	hRf Values	No. of Com	hRf values	No. of Corn	hRf values
	40:60	4	67,78,85,92	4	10,17.64,75	0	0
Benz/MeOH	50:50	5	4956,59,72,80	3	13,34,65	0	0
	60:40	6	17,65,22,89,93,96	2	52,59	0	0
	40.60	2	56,91	3	11,56,88	1	13
$CHCl_3$/MeOH	50:50	3	14,46,82	2	32,65	1	23
	60:40	4	29,47,65,91	1	14	1	14
	40:60	5	35,50,64,78,89	6	7,16,28,46,	6	4,23,35,60,
					75,89		67,85,
Ethyl acetate/	50:50	3	13,78,88	2	9,68	3	3,78,84
MeOH	60.40	4	64,67,70,89	4	67,70,76,85	4	6,23,64,87
	40:60	2	78,92	1	79	1	89
Acetic acid/	50:50	2	76,86	1	73	1	86
MeOH	60:40	2	75,85	1	75	1	82
	40:60	1	79	1	78	1	87
McOH/HCl	50:50	1	73	1	73	1	85
	60:40	I	75	1	72	1	87

Table-3
Physico-chemical Characteristics of Purified Compounds isolated from the Methanol Extract of Roots of *S. spelendens*

No.	Characteristics	Compound CI	Compound C2	Compound C3
1	Colour	Brownish Yellow	Yellow	Light Yellow
2	Physical State	Thick Viscous	Thick Viscous	Thick Viscous
3	Fluorescence	Pinkish	Pinkish	Pinkish
4	Reaction with iodine	Yellow	Yellow	Yellow
5	Reaction with o-Dianisidene[s]	Red colour (Ketonic group)	Red colour (Ketonic group)	Rod colour (Ketonic group)
6	Reaction with Nitroprusside (sodium)NaOH (Legal Test)	Violet colour (Ketonic group)	Pink colour (Aldehydic group)	---
7	Reaction with Hydroxyamine/ ferric chloride**	---	Brown colour (Phenolic group)	Dark brown (Tannin)

No.	Characteristics	Compound C1	Compound C2	Compound C3
8	Reaction with $SbC1_3$	Yellow ppt.(Sterol), Terpene)	---	---
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(2),1390(m), 1470(s),1390(m), 725 (w)

*'Saturated solution of0-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 25 ml w/v).
Solution II. 1 g $FeCl_3$ in 2 ml 36 % HCl w/v + 20 ml $(CH_3)_2O$
Both the solutions were mixed before use.

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 C1	Compound C2	Compound C3	Tetracycline
Gram +ve Bacteria	36.55±2.04	22.352431	----	----	42.35±3.50
Bacillus pumilus					
Bacillus subtilis	20.00±1.03	1.00±2.13	6.00±1.25	8.50+0.41	25.05+2.41
Staphylococcus aureus	38.65±1.67	5.50±3.51	9.00 ± 1.04	26.40±3.7	42.35±5.60
Gram -ve Bacteria					
Proteus vulgaris	32.45±025	12.50±2.50	----	22.25±3.4	42.35±5.70
Escherichia colt	21.25±1.01	10.00±1.50	----	----	26.15±320
Pseudomonas aeruginasa	22.00±2.0	10.00±1.666	13.00±2.2	----	41.30± 4.21
Fungus *Candida albicans*	25.35±4.80	----	15.25 ± 2.3	35.55±5.41	32.55±5.26

*Mean values of six replicates with standard error shown as ±

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