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BIOLOGICAL SCREENING OF *SALVIA CABULICA*

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Abstract

In the present study, *Salvia cabulica* an endemic plant of Balochistan, was screened for different biological activities for the first time. For this purpose, 6 crude extracts i.e., petroleum ether (A), dichloromethane (B), ethyl acetate (C), acetone (D), butanol (E) and aqueous (F) (Fig. 1) were obtained from 80% ethanolic extract of the dry plant of *Salvia cabulica* and screened for antibacterial, antifungal, phytotoxic and insecticidal activities. Extracts B, D and E showed low antibacterial activities against *Salmonella typhi*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However extracts A, E and F showed moderate antifungal activity against *Trichophyton longifusis* and *Microsporum canis*, while extract B showed significant phytotoxicity and insecticidal activities.

Introduction

Salvia, the largest genus of the family Lamiaceae, includes about 900 species widely distributed all over the world (Tawfeq, 2003; Chada, 1972). It comprises of aromatic and ornamental herbs and shrubs distributed in tropical and temperate regions. The two largest centers of this genus are in America and in South West Asia (Hedge, 1960; 1992). Turkey is the major center for the genus *Salvia* with a number of endemic species (Davis & Mill, 1988; Vural & Adiguezel, 1996). Sixteen species are found in Pakistan (Nasir & Ali, 1990).

Salvia has always been an important group of useful plants since ancient times; active constituents of some species are reported for its curative properties (Huang, 1991). *Salvia* species contain antiseptic monoterpenes (Nakipoglu, 1993), diterpenes, flavones, flavone glycosides, anthocyanins and proanthocyanins, hence used particularly as spasmolytic, antiseptic, astringent (Newall & Anderson, 1996), anti neurasthenic insomnia, antitumor (Ulubelen & Topcu, 1992), antidiabetic (Dobrynin & Kolosov, 1976) etc. Other biological activities have also been reported, such as antibacterial (Janssen & Chin, 1986-88; Gonzalez & Abad, 1989; Ulubelen & Topcu, 1998a, b, 1994; Miski & Ulubelen, 1983), antituberculous, cytostatic (Ulubelen & Topcu, 1997), antiviral (Darias & Bravo, 1990) and antioxidant activities (Tada & Okuno, 1994; Nakatani, 1994; Lu & Foo, 2002; Mantle & Pickering, 2000). *Salvia* has glandular hairs, which contain fragrant ether like essential oils, a characteristic feature of most of the species; therefore it is widely used in perfumery and as sweetener in the food industry (Kesercioglu & Nakipoglu, 1992).

Salvia cabulica is an aromatic shrub with lilac flowers, branched and aromatic leaves with hairy stem. The plant is found in dry rocky hills (Nasir & Ali, 1990) at an altitude of 1600 – 2400m (Yasin & Rubina, 1995). It is widely distributed in Afghanistan and Pakistan (Marilee & Shah, 1997), particularly in various regions of Balochistan such as Urak, Wali tangi (Quetta), Mach, Loralli, Ziarat, Bolan Pass and in the Murdar range (Nasir & Ali, 1990; Shareeque Khan, 1998). The decoction of the plant is reported to be used as flu fighter and as a cure for lung diseases in folk medicines (Shareeque Khan, 1998).

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In the present study, crude extracts A, B, C, D, E and F (Fig. 1) from 80% ethanolic extract of *S. cabulica* were screened for different biological activities. Extract B appeared to have significant phytotoxic and insecticidal activities against *Lemna* minor and *Tribolium castaneum*, respectively, while other extracts A, C, D, E & F showed variable response in these assays.

Material and Methods

1. Plant material: The aerial part of wildly grown plant *S. cabulica* was collected during the flowering period from Mach and Ziarat, Balochistan, Pakistan in June 2005. Herbarium samples were prepared and identified by Prof. Dr. M. Qaiser, of the Botany Department, University of Karachi (No.68506) and a voucher specimen was deposited in the Herbarium of University of Karachi. Dried sample was used for further proceedings like biological screening.

2. Morphological properties: *Salvia cabulica* is an aromatic shrub, morphological properties of its root, stem, leaf and flower are given below:

Root: The dense dark brown hard bark surrounds the root.

Stem: The herbaceous stem is 80 cm long, erect and the upper part of the stem is covered by the eglandular villous, glandular hairs and numerous sessile oil globules which contain essential oils.

Leaf: Leaves are simple and mostly basal. Glandular and eglandular hairs are present on both the upper and lower epidermis. The petiole is 20 mm long.

Flower: Inflorescence is inconspicuous and flowers are verticillate. Bracts are 5x2 cm long and broadly ovate. The shape of calyx is tubular- companulate. Calyx is 10 mm long and has a dense indumentums of capitates glandular hairs. The corolla is 18 - 25 mm long, has bluish violet colour. Corolla tube is 15 mm in length. Fruit type is nut let and seeds are brown in colour.

3. Extraction: The air dried plant material of *Salvia cabulica* (2.5 Kg) was soaked in 80% ethanol and it was kept at room temperature for two weeks and concentrated on rotary evaporator under reduced pressure, dark green gummy residue (120 g) was obtained. The gummy residue was dissolved in distilled water to make a suspension and it was defatted first with petroleum ether and then extracted with different solvents such as dichloromethane, ethyl acetate and butanol respectively. The insoluble portion left over was dissolved in acetone. Six extracts i.e., petroleum ether (A), dichloromethane (B), ethyl acetate (C), acetone (D), aqueous (E) and butanol (F) were obtained that were investigated for different biological activities (Fig. 1).

4. Test organisms: *Escherichia coli*, *Bacillus subtilis*, *Shigella flexaneri*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi* were used as bacteria *Trichophyton longifusis* and *Fusarium solanivar* plant pathogens, human pathogens *Candida albicans* and *Aspergillus flavus* and animal pathogen *Microsporum canis*, as fungi for testing the antibacterial and antifungal activities respectively. Bacteria and yeast were obtained from the standard bacteria and fungus strain.

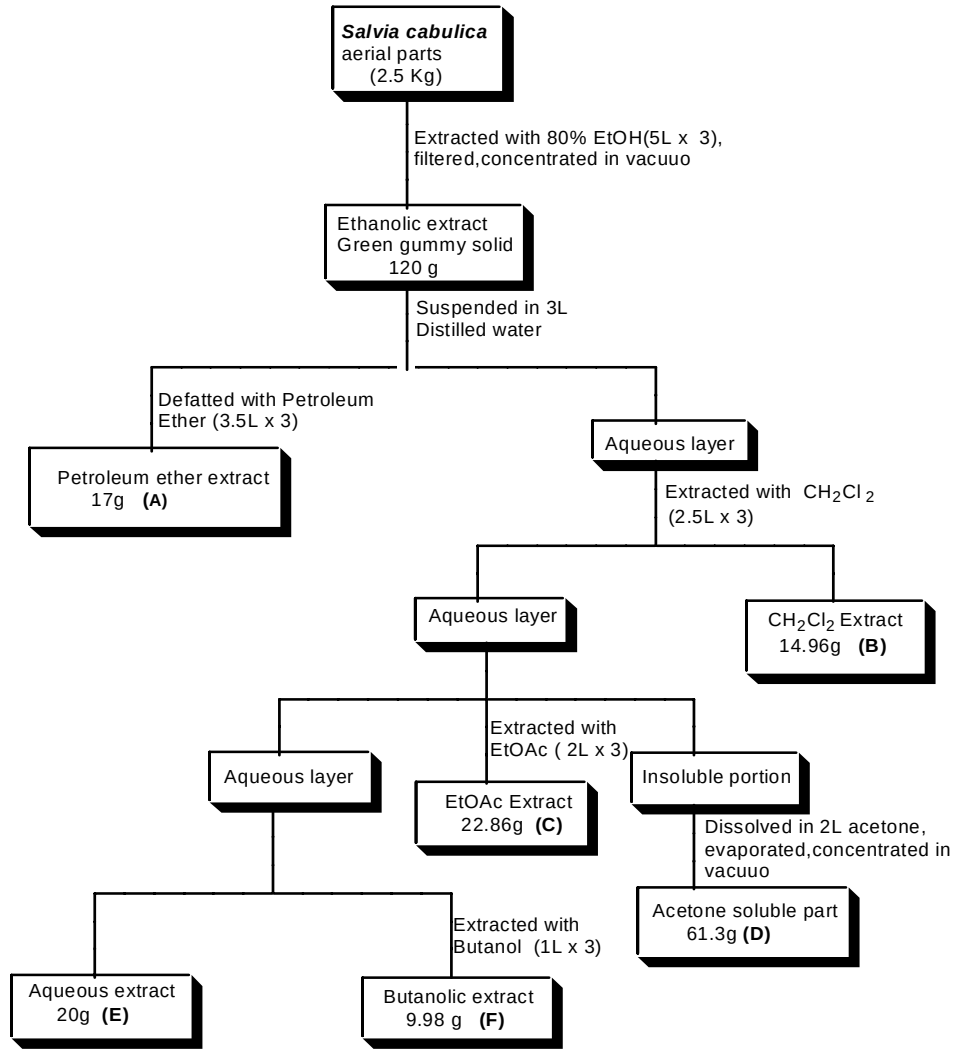

Fig. 1. Extraction of ethanolic extract of *Salvia cabulica*.

The stored grain pests viz., *Tribolium castaneum* (Red flour beetle), *Callosbruchus analis* (Pulse beetle), *Sitophilus oryzea* (Rice weevil) and *Rhyzopertha dominica* (Lesser grain borer) were used for the evaluation of insecticidal activity.

5. Biological activities

i Antibacterial activity: The extracts A, B, C, D, E and F (Fig. 1) obtained from the crude ethanolic extract of *S. cabulica*, were used to study the biological activities against several pathogenic bacteria. The agar well diffusion method was used for a preliminary, qualitative evaluation of antimicrobial activity. Overnight cultures of bacteria viz., *Escherichia coli, Bacillus subtilis, Shigella flexaneri, Staphylococcus aureus,*

Pseudomonas aeruginosa and *Salmonella typhi* were developed. Then bacterial lawn was made on nutrient agar plates by dispensing soft agar containing 100 µl cultures and allowed to be solidified. Wells were made by 6 mm sterile metallic borer. The crude fractions were dissolved in DMSO (3 mg/ 3 ml) and further dilution was made with deionized distilled water and 100 µl of different samples was loaded in labelled wells. The plates were incubated for 24 hours at 30°C. Imipenum (standard drug) and DMSO were used as positive and negative controls. The amount of growth in each well was determined visually by comparing with the growth in the control wells (Jorgensen & Turnidge, 1999; Stepanovic & Anetic, 2003; Carron & Maran, 1987; Bektas & Donmez, 2004; Kivack & Mert, 2002).

ii. Antifungal activity: In the preliminary stage of the antifungal bioassay, the antifungal activities of petroleum ether (A), dichloromethane (B), ethyl acetate (C), acetone (D), aqueous (E) and butanol (F) extracts (Fig. 1), obtained from the extract of *S. cabulica*, were screened against different fungal pathogens viz., *Trichophyton longifusis, Candida albicans, Aspergillus flavus, Microsporum canis* and *Fusarium solanivar*. Agar tube dilution method was used for the evaluation of their antifungal activity. Sabouraud dextrose agar (SDA) was used for the growth of fungus and stock solution was prepared by dissolving 24 mg of each extract in 1 ml of sterile DMSO. Acidic Media (pH 5.5- 5.6), containing high concentration of glucose or maltose was prepared by mixing 32.5 gm/500 ml of distilled water. It was kept in the screw caped tubes and autoclaved at 121°C for 15 minutes. Tubes were then allowed to cool to 50°C. The non-solidified SDA was loaded with 66.6 µl of extract, pipetted from the stock solution and allowed to solidify at room temperature. Then tubes were inoculated with 4mm piece of inoculums, incubated at 27-29° C for 7-10 days relative humidity of incubation room maintaining at 40-50%. After this period, percentage growth inhibition was calculated with reference to the negative control by applying the formula: % inhibition = linear growth in test (mm)/ linear growth in control (mm) x 100. Miconazole and amphotericin B were used as standard drugs, while miconazole, amphotericin B and DMSO were used as positive and negative controls (Alves & Silva, 2000; Berhge & Vlientinck, 1991; Choudhary & Dur-e-Shawar, 1995; Janaki & Vijayasekaran, 1998; Peters & Gills, 1995).

iii. Phytotoxic activity: Phtotoxicity of the crude extracts A, B, C, D, E & F (Fig. 1), were tested against *Lemna minor*. It is a floating aquatic plant, used as a tool to monitor the effects of different bioactive constituents present in the crude extracts as inhibitors or stimulators. The selected method consisted mainly of *Lemna minor* and E-medium. The stock solution of E-medium was prepared by the addition of Potassium dihydrogen phosphate (0.68 g), Potassium nitrate (1.515 g), Calcium nitrate (1.180 g), Magnesium sulphate (0.492 g), Boric acid (0.00286 g), Ferric chloride (0.00540 g), Zinc sulphate (0.00022 g), Copper sulphate (0.00022 g), Sodium molybdate (0.00012 g), Ethylene diamino tetra acetic acid (0.01120 g) in 1L of distilled water and pH was adjusted between 6.0 and 7.0 by adding KOH pellets. Further dilution was made by addition of 100 ml of stock solution with 900 ml distilled water. Plants were grown in 25 ml flasks containing specifically prepared E- medium in growth cabinet at 30°C at 50–60% relative humidity for 7days. Then 30 mg of crude fractions were dissolved in 1.5 ml of methanol, further dilution was made by adding distilled water and three flasks were inoculated with 10,100 and 1000 µg/ml of the extract. Ten plants with three fronds each were placed in each flask and incubated at 30°C, the number of fronds in each flask was counted after 7

days and their percentage growth regulation was calculated by applying the following formula:

$$\% \text{ Regulation} = \frac{100 - \text{No. of fronds in test}}{\text{No. of fronds in –ve control}} \text{ X } 100$$

The result was calculated with reference to the positive and negative control. Paraquat was used as a standard drug, while paraquat and volatile solvent were used as positive and negative controls (Atta-ur - Rahman, 1991; Lewis, 1995; Finny, 1971; Hideji & Oshida, 1982).

iv. Insecticidal activity: The extracts A, B, C, D, E and F (Fig. 1), obtained from the extraction of *S. cabulica,* were evaluated against different insects viz., *Tribolium castaneum*, C*allosbruchus analis, Sitophilus oryzea* and *Rhyzopertha dominica*. The test sample was prepared by dissolving 200 mg of crude fractions in 3 ml acetone and loaded in a Petri dishes covered with the filter papers. After 24 hours, 10 test insects were placed in each plate and incubated at 27^{o}C for 24 hours with 50% relative humidity in growth chamber. The results were analyzed as percentage mortality, calculated with reference to the positive and negative controls. Permethrin was used as a standard drug, while Permethrin, acetone and test insects were used as positive and negative controls (Collins, 1998; Tabassum & Naqvi, 1997; Atta-ur-Rahman & M. I. Choudhary, 2001; Abbott, 1925). The percentage mortality was calculated by the formula:

$$\frac{\text{100- Number of insects alive in test}}{\text{Number of insects alive in control}} \text{ X } 100$$

Results and Discussion

The petroleum ether (A), dichloromethane (B), ethyl acetate (C), acetone (D), aqueous (E) and butanol (F) extracts (Fig. 1), obtained from the extraction of *S. cabulica*, were screened for antibacterial, antifungal, phytotoxic and insecticidal activities. Different biological screening of crude extracts A, B, C, D, E and F were proved to be significant in some extracts, while other extracts showed variable response for these bioassays.

Different bacterial isolates comprising of both Gram negative and Gram positive organisms were used for the evaluation of antibacterial activity. The result showed that extracts from *S. cabulica* (aerial parts) possessed *In vitro* antibacterial activity, although they differ significantly in their activities against tested micro organisms. The acetone (D) and aqueous (E) extracts proved considerably more active than dichloromethan extract (B). Maximum antibacterial activity was shown by acetone (D) and butanol (E) extracts against *S. aureus* and *P. aureginosa*. Antibacterial activity of *S.cabulica* is shown in Table 1 & Fig. 2. Neither pet ether (A) extract nor butanol (F) extract was able to inhibit any of the tested bacterial strain.

As far as the antifungal activity is concerned, extract A showed moderate antifungal activity against *Trichophyton longifusis*, extract E showed moderate activity against *Trichophyton longifusis* and *Microsporum canis*, whereas extracts B, C and D appeared to be non significant (Table 2 & Fig. 3).

Table 1. *In vitro* Antibacterial activity (MIC)[a] of Crude fractions (3 mg/3 ml of DMSO) of *S. cabulica*.

Bacterial species	Zone of inhibition of std. drug	Zone of inhibition of different samples [b]					
		A	B	C	D	E	F
E. coli	30	--[c]	09	10	10	--[c]	10
B. subtilis	33	09	--[c]	--[c]	10	--[c]	--[c]
S. flexaneri	27	--[c]	11	--[c]	--[c]	--[c]	--[c]
S. aureus	45	--[c]	11	--[c]	14	--[c]	--[c]
P. aeruginosa	24	--[c]	09	--[c]	--[c]	15	--[c]
S. typhi	25	10	12	10	--[c]	--[c]	12

a. Minimal inhibitory concentration given in mg/ml.
b. Zone of inhibition taken in mm (<9 mm: not activity, 9-12: Non- significant, 13-15 mm: low activity, 16-18 mm: good activity, >18 mm: significant activity).
c. Not active.

Table 2. Antifungal activities (MIC µg ml^{-1}) of Crude fractions (400 mg ml^{-1}) of aerial parts of *S. cabulica*.

Fungal species	Miconazole % inhibition of Std. drug	% Inhibition of different samples					
		A	B	C	D	E	F
T. longifusis	70	50	20	20	40	60	30
C. albicans	110.8	--[a]	--[a]	--[a]	--[a]	--[a]	--[a]
A. flavus	20	--[a]	--[a]	--[a]	--[a]	--[a]	--[a]
M. canis	98.4	30	--[a]	--[a]	--[a]	30	--[a]
F. solanivar	73	--[a]	--[a]	--[a]	--[a]	20	--[a]
C. glaberata	110.8	--[a]	--[a]	--[a]	--[a]	--[a]	--[a]

a. Not active.
b. In case of *Aspergillus flavus*, amphotericin B was used as standard drug instead of Miconazole

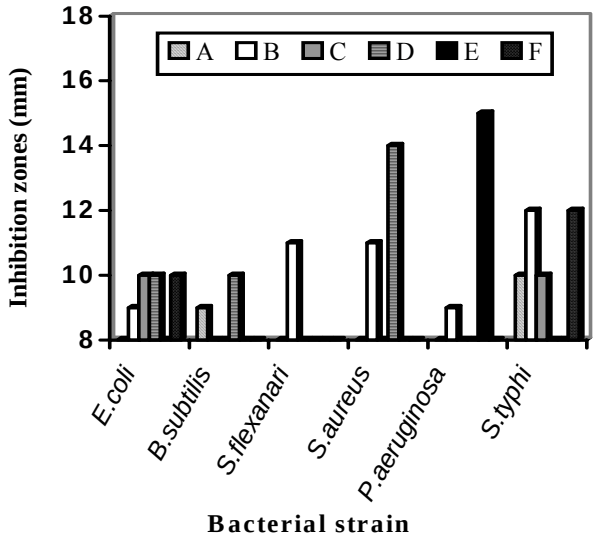

Fig. 2. Antibacterial activity of *S. cabulica*.

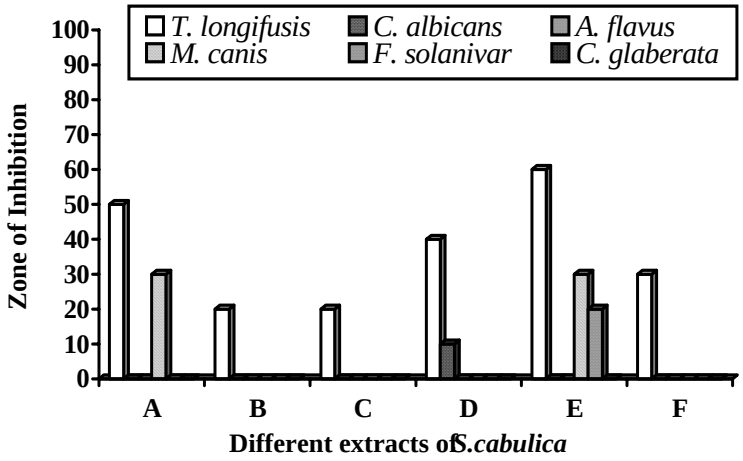

Fig. 3. Antifungal activity of different extracts of *S. cabulica*.

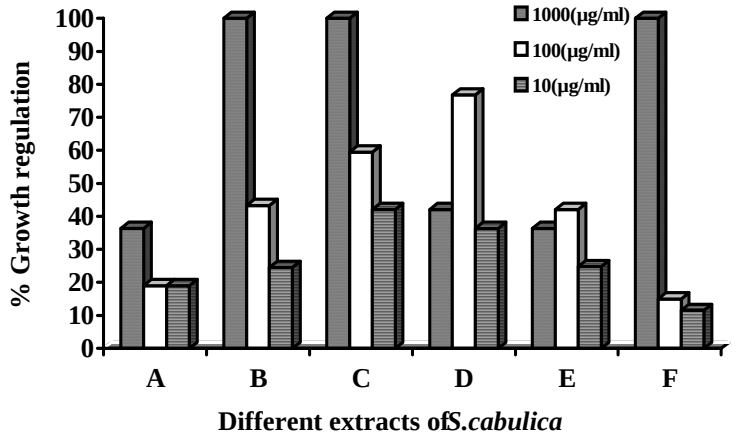

Fig. 4. Antifungal activities of extracts A-F.

The extracts B, C, D & E were significantly phytotoxic against *Lemna minor* and it was inferred from experiments about its dose dependency (Table 3 & Fig. 4) i.e., at high concentration is highly significant and the activity decreased with decrease in concentration. The butanol extract (F) showed moderate activity at high concentration. This activity is relatively similar to the standard drug paraquat and indicates the presence of herbicidal compound in extracts B, C, D & E. A number of polyphenolic compounds and tannins are reported in genus *Salvia*. The presence of these compounds, which are toxic, can be another reason for the death of host tissues as these toxins can easily penetrates into the host cells. The Phenolic compounds can mediate harmful interactions directly or indirectly by linking autotrophs to each other and to herbivores (Waterman & Mole, 1994).

Table 3. *In vitro* phytotoxic bioassay of different fractions of *S. cabulica.*

Samples	Conc. of different fractions	No. of fronds samples	Control	% Growth regulation	Conc. of std. drug (µg/ml)
A	1000	11	17.3	36.4	0.015
B		0	10.6	100	
C		0	17.3	100	
D		10	17.3	42.1	
E		11	17.3	36.4	
F		0	11.3	100	
A	100	14	17.3	19.0	0.015
B		06	10.6	43.3	
C		07	17.3	59.5	
D		04	17.3	76.8	
E		10	17.3	42.1	
F		13	11.3	15.0	
A	10	14	17.3	19.0	0.015
B		08	10.6	24.5	
C		10	17.3	42.1	
D		11	17.3	36.4	
E		13	17.3	24.8	
F		10	11.3	11.5	

a. Lemna minor was used to investigate the phytotoxic activity
b. Incubation condition: 28 ± 1°C.

Table 4. Insecticidal activity of crude extracts of *S. cabulica* by contact toxicity method.

Fungal species	% Mortality		% Mortality of samples					
	+ve Control	-ve Control	A	B	C	D	E	F
T. castaneum	100	0	NT	80	NA	NA	NA	NA
S. oryzae	100	0	NT	NT	NA	NA	NA	NA
R. dominica	100	0	NT	NT	NA	NA	NT	NA
C. analis	100	0	NT	20	NT	NT	NT	NT

+ve control: contains standard insecticide (Permethrin) and test insects
-ve control: contains volatile solvents and test insects.
NT: not tested; NA: not active.
Minimal concentration of sample is 1572 µg cm^{-2} and standard drug is 393.17 µg cm^{-2}.

The crude dichloromethane (B) extract of *S. cabulica* exhibited significant insecticidal activity against *Tribolium castaneum* and low activity against *Callosbruchus analis*, while other extracts i.e., A, C, D, E & F appeared non significant against *Tribolium castaneum* and *Sitophilus oryzae*. Results are presented in Table 4.

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